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TITLE: Preclinical Validation of Anti-Nuclear Factor Kappa B Therapy against Vestibular Schwannoma and Neurofibromatosis Type II

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**14. ABSTRACT**

**Background:** Neurofibromatosis type 2 (NF2) is a genetic disorder that causes substantial suffering and debility due to many tumors that occur on the nerves within the skull and spine throughout a person's life. The hallmark of NF2 is vestibular schwannomas (VSs), also known as acoustic neuromas, which occur on the vestibular nerves that connect the inner ear with the brain. Initially, VSs cause hearing loss. However, as they grow, they can compress the brainstem and cause death. Current treatment options are limited to surgical removal and radiation therapy, both of which carry substantial risks, including deafness and facial paralysis. Although drug therapies against NF2 are gaining momentum, more effective and better tolerated drugs are sorely needed. Because NF2 tumors are typically slowly growing and non-malignant, even therapies that simply reduce tumor volume and retard growth can be life-saving. The most successful drug used today to treat NF2, bevacizumab, works in only about 50% of patients in halting tumor growth or causing tumor shrinkage. Bevacizumab is known to inhibit vascular endothelial growth factor (VEGF), but its precise mechanism of action in VSs is unknown.

**Progress:** Our overriding objective is to develop new and better drug therapies to help people with NF2. Using an unbiased bioinformatic approach that synthesizes published knowledge on the genes that are known to be aberrantly expressed in NF2, we have identified a key role for nuclear factor kappa B (NFκB). We hypothesize that increased NFκB signaling in VS contributes to abnormal growth, and that inhibition of the NFκB pathway can prevent growth and promote death of VSs. We have proven this hypothesis *in vitro*, using primary human VS cells treated with 3 different NFκB inhibitors: (1) shRNA, (2) an experimental drug, BAY11, and (3) a dietary supplement, curcumin. Since NFκB is a major regulator of inflammation, we have also tested the hypothesis that non-steroidal anti-inflammatory drugs (NSAIDs) inhibit VS proliferation *in vitro*. We have proven the hypothesis using 3 commonly used and well tolerated NSAIDs: aspirin, sodium salicylate and 5-aminosalicylic acid. These data corroborate our published finding of the association between aspirin intake and halted growth of VS in patients *in vivo*. We have further demonstrated cross-talk between VEGF and cMET signaling pathways in human VSs *in vitro*, which highlights the hepatocyte growth factor (HGF)-cMET pathway as an additional important therapeutic target in VSs.

**Planned experiments:** Encouraged by our results *in vitro*, we will now focus on experiments *in vivo* to further test the hypothesis that inhibition of the NFκB pathway can prevent growth and promote death of VSs. We will test the efficacy of NFκB inhibition alone or in combination with bevacizumab as new treatments for NF2. Our animal model is immunocompromised mice that can receive human VS xenograft without rejecting it. The mice will receive curcumin, or bevacizumab, or both, or neither. We will monitor tumor growth and response to therapy in alive mice through blood assays and bioluminescence imaging.

**Applicability:** Our work is applicable for patients with stable or progressive NF2 disease, with a particular focus on VSs, hallmark tumors of NF2. Our work also has implications for the closely related diseases such as schwannomatosis, which sometimes manifests with VS, and sporadic VS, which affects vestibular nerves on one side only. Drug therapies for sporadic VS are virtually non-existent. More broadly, our work may affect treatment of patients with cancers that have increased activity of NFκB and VEGF, such as breast, colon and ovarian cancer.

**Contributions – existing and potential:** Our results thus far have stimulated the design of a new clinical trial to prevent VS growth with anti-inflammatory medications such as aspirin. If our proposed experiments *in vivo* support our findings *in vitro* that curcumin inhibits VS proliferation, this will stimulate a clinical trial with curcumin because it is extremely well tolerated when taken as a dietary supplement or therapy for several types of disease. By delineating what, if any, fraction of bevacizumab's beneficial effect is via NFκB signaling, and by testing combination therapy against NFκB and VEGF for human cells, we set the stage for a future clinical trial using combination therapy targeting NFκB and VEGF, a concept that is widely accepted in the context of cancer but has yet to be applied to NF2 treatment.

**15. SUBJECT TERMS**

None Listed

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## **1. INTRODUCTION**

**Background:** Neurofibromatosis type 2 (NF2) is a genetic disorder that causes substantial suffering and debility. Current treatment options are limited to surgical resection and stereotactic radiation therapy, both of which carry substantial risks, including deafness and facial paralysis. Although pharmacotherapies against NF2 are gaining momentum, the most successful so far, namely a vascular endothelial growth factor (VEGF) inhibitor bevacizumab, halts or retards tumor growth in only 54% of the patients, and can also cause significant side effects such as renal failure. Pharmacologic treatment of NF2 represents a major unmet medical need. The hallmark of NF2 is bilateral vestibular schwannomas (VSs), which arise from the vestibular nerves. Although VSs typically cause hearing loss, they can lead to death from brainstem compression. We focus on VS as a model NF2 tumor to develop well tolerated pharmacotherapies to treat NF2.

**Objective/Hypothesis:** Using a systems biology-based bioinformatic analysis of all genes implicated in VS pathobiology, we found nuclear factor kappa B (NF $\kappa$ B), a pro-inflammatory transcription factor, to be aberrantly upregulated, translated and activated in VS in comparison to healthy nerves. Although novel in NF2 and VS, NF $\kappa$ B is known to play a major role in various other neoplasms. Our **leading hypothesis** is that upregulation of NF $\kappa$ B signaling and inflammation in VS contributes to neoplastic growth, and that specific targeting of the NF $\kappa$ B pathway and inflammation can be therapeutic against VS. Thus far we have proven the hypothesis *in vitro* (**Aim 1**), using primary human VS cells and the human NF2 VS cell line compared to control primary human Schwann cells. These cells were treated with (1) siRNA, (2) an experimental drug, BAY11, or (3) a dietary supplement, curcumin. We now plan to focus on testing the hypothesis *in vivo*, using human VS xenografts on the sciatic nerve in nude mice (**Aim 3**). We have also discovered an additional therapeutic target in VS: HGF (hepatocyte growth factor)-cMET pathway, which interacts with the VEGF pathway (**Aim 2**). Our ultimate objective is to develop novel and effective pharmacologic strategies to treat NF2 and VS.

### **Specific Aims:**

**Aim 1:** Define parameters for and therapeutic specificity of NF $\kappa$ B inhibition in human VS cells without causing cytotoxicity in healthy human Schwann cells *in vitro*.

- This has been completed and two papers have been published.

**Aim 2:** Determine whether therapeutic VEGF inhibition in VS cells acts solely through the VEGFR pathway *in vitro*.

- This has been completed and a paper has been published.

**Aim 3:** Test efficacy of NF $\kappa$ B inhibition *in vivo* using primary human VS cells xenografted onto the sciatic nerve of nude mice.

- This will be our focus over the next 2 years.

**Innovation:** Our focus on NF $\kappa$ B as a potent pharmacologic target for NF2 is novel. Our work emphasizes the role of pathological inflammation in NF2 and VS, an aspect that has not been explored but could be a major modulator of the disease. We use NF $\kappa$ B inhibitors that are novel to the NF2 and VS fields: BAY 11, an experimental drug, and curcumin, a dietary supplement. Our exploration of cocktail pharmacotherapy for NF2 is innovative, and has a potential to prevent resistance to and toxicity of bevacizumab through synergy with curcumin. Unlike many pharmacotherapies in clinical trials against NF2 today, curcumin is extremely well tolerated, and effective against several malignancies.

## **2. KEYWORDS (and ABBREVIATIONS)**

- 5-ASA: 5-aminosalicylic acid
- BAY11: BAY11-7082)
- cMET: MNNG HOS transforming gene
- COX2: cyclooxygenase 2
- GAN: Great auricular nerve
- HGF: Hepatocytes growth factor
- I $\kappa$ B $\alpha$ : Inhibitor of kappa B alpha
- MET: gene encoding cMET protein
- NaSal: sodium salicylate
- NF2: Neurofibromatosis type 2

- NF-κB: Nuclear factor kappa B
- PTG: prostaglandin
- SC: Schwann cell
- siRNA: small interfering RNA
- S100: Schwann/schannoma cell marker
- TNF: Tumor necrosis factor
- VEGF: Vascular endothelial growth factor
- VEGFR: vascular endothelial growth factor receptor
- VS: Vestibular schwannoma

### **3. ACCOMPLISHMENTS**

#### **3.1 Activities in relation to statement of work.**

***Aim 1: Define parameters for and therapeutic specificity of NFκB inhibition in human VS without causing cytotoxicity in healthy human Schwann cells in vitro.***

Our bioinformatic network analysis of all genes reported to be differentially expressed in human VS revealed a pro-inflammatory transcription factor nuclear factor-kappa B (NF-κB) as a central molecule in VS pathobiology. Assessed at the transcriptional and translational level, we found canonical NF-κB complex to be aberrantly activated in human VS and derived VS cultures in comparison to control nerves and Schwann cells, respectively. We therefore treated cultured primary VS cells and VS-derived human cell line HEI-193 with specific NF-κB siRNAs, experimental NFκB inhibitor BAY11-7082 (BAY11) and clinically relevant NF-κB inhibitor curcumin. Healthy human control Schwann cells from the great auricular nerve were also treated with BAY11 and curcumin to assess toxicity. All three treatments significantly reduced proliferation in primary VS cultures and HEI-193 cells, with siRNA, 5 mM BAY11 and 50 mM curcumin reducing average proliferation ( $\pm$  standard error of mean) to  $62.33\% \pm 10.59\%$ ,  $14.3 \pm 9.7\%$ , and  $23.0 \pm 20.9\%$  of control primary VS cells, respectively. These treatments also induced substantial cell death. Curcumin, unlike BAY11, also affected primary Schwann cells. This work highlights NF-κB as a key modulator in VS cell proliferation and survival and demonstrates therapeutic efficacy of directly targeting NF-κB in VS. This work has been published in *Molecular Oncology*, and presented at the Children's Tumor Foundation meeting (see section 6 and Appendix 9.1 for details).

Because of NFκB's pro-inflammatory role, we tested whether another group of anti-inflammatory medications may be therapeutic against VS. We focused on non-steroidal anti-inflammatory drugs (NSAIDs) because they are well tolerated and widely clinically used for other indications, and we explored their repurposing for VS. We found COX-2 to be aberrantly expressed in human VS and primary human VS cells in comparison with control human nerve specimens and primary Schwann cells (SCs), respectively. Furthermore, levels of prostaglandin E2, the downstream enzymatic product of COX-2, were correlated with primary VS culture proliferation rate. We then treated VS cultures with 3 clinically used non-steroidal anti-inflammatory drugs (NSAIDs) that act via COX2 inhibition: aspirin, sodium salicylate or 5-aminosalicylic acid. These drugs decreased proliferation and viability of VS cells without affecting their death rate; healthy Schwann cells were not affected. The cytostatic effect of aspirin *in vitro* was in concurrence with our previous retrospective clinical finding that patients with VS taking aspirin demonstrate reduced tumor growth. Overall, this work suggests that COX-2 is a key modulator in VS cell proliferation and survival and highlights salicylates as promising pharmacotherapies against VS. This work has been published in *Translational Research*, and presented at the Children's Tumor Foundation meeting and the meeting of the Association for Research in Otolaryngology (see section 6 and Appendix 9.2 for details). Results of this study are motivating the design of a prospective randomized clinical trial to determine whether aspirin or related COX-2 inhibitor can prevent growth of VSs in patients with sporadic and NF2 associated tumors.

***Aim 2: Determine whether therapeutic VEGF inhibition in VS cells acts solely through the VEGFR pathway in vitro.***

This aim differs slightly from our originally proposed aim to determine whether therapeutic VEGF inhibition in VS cells acts solely through the NF $\kappa$ B pathway *in vitro*. We found that pharmacologic inhibition of VEGF with bevacizumab in cultured human VS cells is not robust. This is in line with the published literature that bevacizumab primarily acts on tumor vasculature, which is not represented in a simplified *in vitro* model of relatively pure Schwann cells.

We therefore focused on investigating, using siRNA, whether VEGF signaling interacts with hepatocyte growth factor (HGF) signaling, an interaction that had been described in other physiological and pathological cell types and may provide a novel therapeutic target. We affirmed previous findings that VEGF-A signaling is aberrantly upregulated in VS, and established that expression of HGF and its receptor cMET is also significantly higher in sporadic VS than in healthy nerves. In primary human VS and Schwann cell cultures, we found that VEGF-A and HGF signaling pathways modulate each other. siRNAs targeting cMET decreased both cMET and VEGF-A protein levels, and siRNAs targeting VEGF-A reduced cMET expression. Additionally, siRNA-mediated knockdown of VEGF-A or cMET and pharmacologic inhibition of cMET decreased cellular proliferation in primary human VS cultures. Our data suggest cross-talk between these 2 prominent pathways in VS and highlight the HGF/cMET pathway as an additional important therapeutic target in VS. This work has been published in *Cancer Biology and Therapy*, and presented at the Children's Tumor Foundation meeting and the meeting of the Association for Research in Otolaryngology (see section 6 and Appendix 9.3 for details).

***Aim 3: Test efficacy of NF $\kappa$ B inhibition in vivo using human VS cells xenografted onto the sciatic nerve of nude mice.***

We have submitted paperwork to the Animal Care Committee of the Massachusetts General Hospital for review. The proposed experiments have already been approved by this committee as a part of a much larger protocol on which Dr. Xandra Breakefield serves as the PI. For simplicity and ease of tracking of our experiments, we have submitted a new animal protocol that covers only experiments proposed in my DoD grant. I serve as the PI on that protocol, which is scheduled to be reviewed on June 17<sup>th</sup>.

**3.2. Opportunities for professional development:**

By attending and presenting at Children's Tumor Foundation meeting for the first time in June 2015 (in Monterey, CA), I had the opportunity to meet and interact with thought leaders in neurofibromatosis research. In addition to presenting two posters, I was invited to give a podium presentation on non-steroidal anti-inflammatory medications being cytostatic against human vestibular schwannomas. Our results thus far are motivating the design of a prospective clinical trial to evaluate whether anti-inflammatory medications, such as aspirin and celecoxib, can prevent growth of VSs.

In addition, I've had the opportunity to interact with international leaders in neurofibromatosis research and clinical care during State of the Art for Neurofibromatosis type 2 meeting in Boston last summer, where I presented a talk on the mechanisms of hearing loss due to VSs.

Also, I have been attending regular seminars in NF2 where I have presented our work in progress in the cross disciplinary forum of Harvard clinicians and investigators with expertise in NF2. This forum includes neurologists, pathologists, geneticists, neurosurgeons, otolaryngologists, virologists and social workers to present a rich opportunity for intellectual cross fertilization.

**3.3. Dissemination of Results**

Results have been disseminated by publications in scientific journals, by presentations at scientific meetings and by media via websites (detailed in Section 6). By presenting at Children's Tumor Foundation, patients and their families were also reached as their representatives attend the meeting, in addition to scientists and clinicians.

**3.4 Plans for the next reporting period**

The efficacy of combination therapy targeting NFκB alongside targeting VEGF with bevacizumab will be explored *in vivo* (**Aim 3**). The *in vivo* model entails growth of primary human VS cells and HEI-193 cells on the sciatic nerve of nude mice. To monitor NFκB activity and tumor burden in real time, all cells will be transduced with two reporters packaged into lentiviruses: (1) NFκB responsive promoter elements driving expression of the secreted *Gaussia* luciferase (Gluc); and (2) a constitutively active CMV promoter driving expression of firefly luciferase (Fluc). Xenografted mice will be injected with curcumin, or bevacizumab, or a combination therapy, or vehicle alone. NFκB activity and tumor growth will be tracked longitudinally, utilizing Gluc-based blood assays and Fluc-based bioluminescence imaging, respectively. Histological studies will define tumor response and systemic toxicity *post mortem*.

#### **4. IMPACT**

Our studies address NFRP's area of emphasis on drug discovery for the treatment of NF2. Our preclinical studies pave the way for rapid translation to clinical trials because we study NFκB inhibitors that include a dietary supplement, curcumin, alone or in combination with bevacizumab, a drug already used in NF2 patients. By studying primary VS, in addition to the NF2 VS cell line, we explore heterogeneity of NF2-related tumors, which is NFRP's area of emphasis. More broadly, our studies have a potential to impact treatment of related diseases, sporadic VS and schwannomatosis, as well as many human malignancies that are characterized by dysregulated NFκB and VEGF signaling.

#### **5. CHANGES/PROBLEMS**

There has not been any major change. The minor changes in Aim 2 are described in section 3.1 above and entail using siRNA to inhibit VEGF signaling and study the cross-talk between it and HGF/cMET signaling. The change occurred because we found that pharmacologic inhibition of VEGF with bevacizumab in cultured human VS cells is not robust, in line with the published literature that bevacizumab primarily acts on tumor vasculature, which is not represented in a simplified *in vitro* model of relatively pure Schwann cells.

#### **6. PRODUCTS**

##### **6.1. Published manuscripts:**

1. Dilwali S, Kao SY, Fujita T, Landegger LD, **Stankovic KM**. Nonsteroidal anti-inflammatory medications are cytostatic against human vestibular schwannomas. *Transl Res*. 2015 Jul;166(1):1-11. doi:10.1016/j.trsl.2014.12.007. Epub 2015 Jan 7.PMC4458444. Acknowledgement of federal support: yes.
2. Dilwali S, Roberts D, **Stankovic KM**. Interplay between VEGF-A and cMET signaling in human vestibular schwannomas and schwann cells. *Cancer Biol Ther*. 2015;16(1):170-5.doi: 10.4161/15384047.2014.972765.PMID: 25692621. Acknowledgement of federal support: yes.
3. Dilwali S, Briët MC, Kao SY, Fujita T, Landegger LD, Platt MP, **Stankovic KM**. Preclinical validation of anti-nuclear factor-kappa B therapy to inhibit human vestibular schwannoma growth. *Mol Oncol*. 2015 Mar 31. pii: S1574-7891(15)00067-8.doi: 10.1016/j.molonc.2015.03.009. [Epub ahead of print] PMID: 25891780. Acknowledgement of federal support: yes.

##### **6.2. Oral presentations:**

1. **Stankovic KM**: Non-steroidal anti-inflammatory medications are cytostatic against human vestibular schwannomas. *Selected oral abstract, Association for Research in Otolaryngology meeting in Baltimore, MD in Feb 2015*
2. **Stankovic KM**: Non-steroidal anti-inflammatory medications are cytostatic against human vestibular schwannomas: from clinic to target and back. *Selected oral abstract, Children's Tumor Foundation Meeting in Monterey, CA, June 2015*.
3. **Stankovic KM** : Exploring salicylates as novel therapy for vestibular schwannoma and neurofibromatosis type 2". *Invited presentation, NIH-National Institute on Deafness and Other Communication Disorders, Bethesda, MD in May 2014*.
4. **Stankovic KM**: Mechanisms of hearing loss due to vestibular schwannoma. *Invited presentation, State of the Art for Neurofibromatosis type 2 conference, Boston, MA in August 2014*.

##### **6.3. Posters:**



1. Dilwali S, Briet M, Kao SY, Fujita T, Landegger L, Platt M, **Stankovic KM**. Pathway and network analysis reveals a central role of NFkB in pathobiology of vestibular schwannoma: preclinical validation. Children's Tumor Foundation Meeting in Monterey, CA, June 2015
2. Dilwali S, Roberts DS, **Stankovic KM**. Interplay between VEGF-A and cMET signaling pathways in human vestibular schwannomas and schwann cells. *Presented at the Association for Research in Otolaryngology meeting in Baltimore, MD in Feb 2015, and at Children's Tumor Foundation Meeting in Monterey, CA, June 2015.*

#### 6.4 Websites that disseminated the results of the research activities:

Press releases about aspirin being cytostatic against human vestibular schwannomas:

- Harvard Medical School news, February 25, 2015: <http://hms.harvard.edu/news/reducing-tumor-growth>
- Medical Express: <http://medicalxpress.com/news/2015-02-salicylates-class-nsaids-vestibular-schwannomas.html>
- EurekAlert: [http://www.eurekalert.org/pub\\_releases/2015-02/meae-sac020515.php](http://www.eurekalert.org/pub_releases/2015-02/meae-sac020515.php)
- News-medical: <http://www.news-medical.net/news/20150209/Salicylates-drugs-reduce-proliferation-viability-of-cultured-vestibular-schwannoma-cells.aspx>
- Audiology online: <http://www.audiologyonline.com/releases/salicylates-class-nsaids-stop-growth-13403>
- Harvard Otolaryngology: <http://issuu.com/hmsotolaryngology/docs/harvard-otolary-spr2015-single/1>

Press releases about the interplay between VEGF-A and cMET signaling pathways in human vestibular schwannomas and schwann cells:

- Massachusetts Eye and Ear: [http://www.masseyeandear.org/news/press\\_releases/recent/2015\\_Stankovic\\_Cancer\\_Biology/](http://www.masseyeandear.org/news/press_releases/recent/2015_Stankovic_Cancer_Biology/)
- News-medical: <http://www.news-medical.net/news/20150221/Researchers-reveal-new-understanding-of-pathobiology-behind-vestibular-schwannoma.aspx>
- Newswise: <http://www.newswise.com/articles/researchers-sheds-new-light-on-biological-pathways-of-vestibular-schwannomas>

#### 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Konstantina Stankovic, MD, PhD
Project Role:	PI
Nearest person month worked:	2
Contribution to Project:	Dr. Stankovic helped with collection of clinical specimens, she designed experiments, oversaw proper data collection and analyses. She wrote papers and presented at scientific meetings.

Name:	Shyan-Yuan Kao, PhD
Project Role:	Post-doctoral fellow
Nearest person month worked:	6
Contribution to Project:	Dr. Kao has performed cell culture experiments

Name:	Takeshi Fujita, MD, PhD
Project Role:	Post-doctoral fellow

Nearest person month worked:	9
Contribution to Project:	<i>Dr. Fujita has helped with the collection of clinical specimens and cell culture experiments.</i>
Funding Support:	<i>Kobe University Graduate School of Medicine, Japan</i>

Name:	<i>Sonam Dilwali</i>
Project Role:	<i>Graduate Student</i>
Nearest person month worked:	3
Contribution to Project:	<i>Sonam Dilwali helped with the collection of clinical specimens and performed cell culture experiments. She also drafted the manuscripts.</i>
Funding Support:	<i>T32 DC00038</i>

Name:	<i>Lukas Landegger</i>
Project Role:	<i>Graduate Student</i>
Nearest person month worked:	1
Contribution to Project:	<i>Lukas Landegger helped with collection of clinical specimens</i>
Funding Support:	<i>Nancy Sayles Day Foundation</i>

**7.2 Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report.

**7.3. Other organizations involved as partners:**

Nothing to report

**8. SPECIAL REPORTING REQUIREMENTS**

None

**9. APPENDICES**

**9.1. Published paper 1:**

Dilwali S, Briët MC, Kao SY, Fujita T, Landegger LD, Platt MP, **Stankovic KM**. Preclinical validation of anti nuclear factor-kappa B therapy to inhibit human vestibular schwannoma growth. *Mol Oncol*. 2015 Mar 31. pii: S1574-7891(15)00067-8.doi: 10.1016/j.molonc.2015.03.009. [Epub ahead of print] PMID: 25891780. Acknowledgement of federal support: yes.

**9.2. Published paper 2:**

Dilwali S, Kao SY, Fujita T, Landegger LD, **Stankovic KM**. Nonsteroidal anti inflammatory medications are cytostatic against human vestibular schwannomas. *Transl Res*. 2015 Jul;166(1):1-11. doi:10.1016/j.trsl.2014.12.007. Epub 2015 Jan 7.PMC4458444. Acknowledgement of federal support: yes.

**9.3. Published paper 3:**

Dilwali S, Roberts D, **Stankovic KM**. Interplay between VEGF-A and cMET signaling in human vestibular schwannomas and schwann cells. *Cancer Biol Ther*. 2015;16(1):170-5.doi: 10.4161/15384047.2014.972765.PMID: 25692621. Acknowledgement of federal support: yes.

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## Preclinical validation of anti-nuclear factor-kappa B therapy to inhibit human vestibular schwannoma growth

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### ABSTRACT

Vestibular schwannomas (VSs), the most common tumors of the cerebellopontine angle, arise from Schwann cells lining the vestibular nerve. Pharmacotherapies against VS are almost non-existent. Although the therapeutic inhibition of inflammatory modulators has been established for other neoplasms, it has not been explored in VS. A bioinformatic network analysis of all genes reported to be differentially expressed in human VS revealed a pro-inflammatory transcription factor nuclear factor-kappa B (NF-κB) as a central molecule in VS pathobiology. Assessed at the transcriptional and translational level, canonical NF-κB complex was aberrantly activated in human VS and derived VS cultures in comparison to control nerves and Schwann cells, respectively. Cultured primary VS cells and VS-derived human cell line HEI-193 were treated with specific NF-κB siRNAs, experimental NF-κB inhibitor BAY11-7082 (BAY11) and clinically relevant NF-κB inhibitor curcumin. Healthy human control Schwann cells from the great auricular nerve were also treated with BAY11 and curcumin to assess toxicity. All three treatments significantly reduced proliferation in primary VS cultures and HEI-193 cells, with siRNA, 5 μM BAY11 and 50 μM curcumin reducing average proliferation (±standard error of mean) to 62.33% ± 10.59%, 14.3 ± 9.7%, and 23.0 ± 20.9% of control primary VS cells, respectively. These treatments also induced substantial cell death. Curcumin, unlike BAY11, also affected primary Schwann cells. This work highlights NF-κB as a key modulator in VS cell proliferation and survival and demonstrates therapeutic efficacy of directly targeting NF-κB in VS.

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Abbreviations: BAY11, BAY 11-7082; GAN, great auricular nerve; IκBα, inhibitor of kappa B alpha; IκK, inhibitor of kappa B alpha kinase; NF-κB, nuclear factor-kappa B; SC, Schwann cell; TNF, tumor necrosis factor; VS, vestibular schwannoma.

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## 1. Introduction

Vestibular schwannomas (VSs) are the fourth most common intracranial tumors (Mahaley et al., 1990). Although histologically non-malignant, they can cause multiple cranial neuropathies and even death due to their location in the cerebellopontine angle and potential for brainstem compression. Currently, main treatment modalities for growing VSs are surgical resection and stereotactic radiotherapy. Although interest in pharmacotherapies against VS is increasing (Plotkin et al., 2012), none are FDA approved. This is partially because drugs such as bevacizumab, which shrink some VSs, have substantial side effects, including renal failure, which may outweigh potential benefits (Plotkin et al., 2012). Therefore, there is an unmet medical need to establish well-tolerated pharmacotherapies to prevent VS growth. Although much is known about the different pathways implicated in VS pathobiology, the interconnectedness among these pathways has not been studied extensively.

To identify the major orchestrators of VS growth, we conducted the first comprehensive network analysis of the published genes aberrantly expressed in sporadic VS. Nuclear factor-kappa B (NF- $\kappa$ B), a transcription factor known for mediating the physiological inflammatory response and pathologic inflammation in several diseases, including neoplastic growth (Hoesel and Schmid, 2013), was identified as a central factor in a top-ranking network. Although NF- $\kappa$ B has been connected to other molecules in VS, NF- $\kappa$ B activation and the accompanying inflammation have not been directly explored as therapeutic targets against sporadic VS. However, level of infiltration of CD163<sup>+</sup> tumor-associated macrophages, known to pathologically promote tumor growth and survival, correlates with human VS growth rate, motivating research to investigate the inflammatory pathways that may promote VS growth (de Vries et al., 2013).

NF- $\kappa$ B can regulate the transcription of over 300 downstream genes, resulting in differential influences on cell growth, proliferation and survival depending on the stimulus (Gilmore, 2014). NF- $\kappa$ B's therapeutic inhibition has been investigated in several cancers because of its role in pathological inflammation accompanying neoplastic growth (Hoesel and Schmid, 2013). NF- $\kappa$ B is especially relevant for VS since merlin, the protein encoded by the NF2 gene, acts as a negative regulator of the NF- $\kappa$ B pathway (Kim et al., 2002) and merlin is dysfunctional in majority of VSs (Lee et al., 2012a,b). Additionally, Axl, a member of the TAM family of receptor tyrosine kinases, regulates overexpression of survivin and cyclin D1 through NF- $\kappa$ B, leading to enhanced survival, cell-matrix adhesion and proliferation of cultured VS cells (Ammoun et al., 2013). NF- $\kappa$ B also regulates p75-associated VS proliferation and apoptosis (Ahmad et al., 2014).

We investigated NF- $\kappa$ B's aberrance in human VS and the therapeutic potential of NF- $\kappa$ B inhibition. Our results suggest that the NF- $\kappa$ B pathway is aberrantly activated in VS and VS-derived cultures compared to healthy nerves and SCs, respectively. NF- $\kappa$ B inhibition in primary VS cells and a VS-derived human cell line using NF- $\kappa$ B siRNA, an experimental NF- $\kappa$ B inhibitor BAY 11-7082 (BAY11) and a clinically

relevant inhibitor curcumin decreased proliferation and survival of the tumor cells. Our work provides novel insight into NF- $\kappa$ B's expression and role in VS pathobiology and demonstrates therapeutic efficacy of directly targeting NF- $\kappa$ B in VS.

## 2. Materials and methods

### 2.1. Ingenuity Pathway Analysis

A literature search was performed with PubMed using MeSH terms neuroma, acoustic, proteins, genes, gene expression, gene expression regulation, gene expression profiling, microarray analysis, DNA mutational analysis, immunohistochemistry, enzyme-linked immunosorbent assay, tumor suppressor proteins, DNA and RNA. Only human studies with relevant controls and explicit description of statistical criteria were selected. Differentially expressed molecules were analyzed on April 14th 2011 using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc.) version 9.0, while setting a cutoff value to 2. Molecules reported to be up- or down-regulated qualitatively were assigned a value 2 or -2, respectively. To avoid a bias toward molecules with extreme differential expression, the absolute maximal value for fold change was set to 100 for molecules with a greater change. The maximal number of molecules per network was 35. The most interconnected molecule in each network is known as the hub.

### 2.2. Specimen collection

Freshly harvested human specimens of sporadic VS and control great auricular nerve (GAN) were collected from indicated surgeries, placed in saline and transported to the laboratory on ice. The study protocols were approved by Human Studies Committee of Massachusetts General Hospital and Massachusetts Eye and Ear Infirmary, and conducted in accordance with the Helsinki Declaration.

### 2.3. Real time quantitative polymerase chain reaction

Expression of genes in the NF- $\kappa$ B pathway was measured using real time quantitative PCR (qPCR). Human VS or GAN tissue was placed in RNA Later (Qiagen) temporarily. RNA was extracted using RNeasy Mini-Kit (Qiagen) and reverse-transcribed to cDNA with Taqman Reverse Transcription Reagent kit (Applied Biosystems), as previously described (Stankovic et al., 2009). qPCR was performed using Applied Biosystems 7700 Sequence Detection System with TaqMan Primers (Applied Biosystems) for NFKB1 (encoding p50 subunit of the NF- $\kappa$ B heterodimer, Hs01042010\_m1), RELA (encoding p65 subunit of the NF- $\kappa$ B heterodimer required for activation, Hs01042010\_m1), TNF (encoding tumor necrosis factor, an inducer for NF- $\kappa$ B, Hs01042010\_m1), RANK (encoding receptor activator of nuclear factor-kappa B, Hs00187192\_m1), NFKB2 (Hs01028901\_g1), REL (Hs00968440\_m1), and RELB (Hs00232399\_m1) and for downstream genes with  $\kappa$ B sites, namely CCND1 (encoding cyclin D1, Hs00765553\_m1), BCL2 (encoding B-cell lymphoma 2, Hs00608023\_m1), CSF2

(encoding colony stimulating factor 2, Hs00929873\_m1), and XIAP (encoding X-linked inhibitor of apoptosis, Hs00745222\_s1). The reference gene was ribosomal RNA 18S (Hs9999901\_s1).

#### 2.4. Protein extraction and quantification

Translation and activation of the NF- $\kappa$ B pathway components were investigated through western blot analysis. Total protein was extracted on ice from freshly harvested specimens of VS and GAN in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche Applied Sciences). The lysate was isolated by centrifugation and stored at  $-80^{\circ}\text{C}$ . Equal protein was loaded per lane, separated on a 4–20% Tris-glycine gel (Invitrogen) and transferred onto a Polyvinylidene fluoride membrane (Millipore). The membrane was blocked and probed with Cell Signaling Technology antibodies against NF- $\kappa$ B phosphorylated (P-) p65 (#3033), NF- $\kappa$ B p65 (#8242), inhibitor of kappa B alpha (I $\kappa$ B $\alpha$ , #11930) or NF- $\kappa$ B p50 (Abcam, #ab7971), followed by secondary antibodies (Jackson-Immuno Research). Membranes were visualized with ChemiDoc XRS+ (Bio-Rad Laboratories). Band densities were quantified using ImageJ and normalized to GAPDH expression (Cell Signaling Technology, #5174).

#### 2.5. Immunohistochemistry

Human VS and GAN specimens were fixed in 4% PFA, transferred to PBS, embedded in paraffin, sectioned, deparaffinized with xylene, washed in PBS, permeabilized with Triton-X 100 (Integra) for 5 min, blocked in normal horse serum and incubated with primary antibodies against S100 (Dako, #Z0311) or p50 (Abcam, #ab7971) and corresponding fluorescent secondary antibodies (Jackson-Immuno Research). Nuclei were labeled with Hoechst stain (Invitrogen). The tissue was visualized and imaged using Carl Zeiss 2000 upright microscope.

#### 2.6. Primary human Schwann cell and vestibular schwannoma cell culture

Using sterile technique, freshly harvested VS or GAN tissue was rinsed in PBS, dissected in culture medium consisting of Dulbecco's Modified Eagle's medium with Ham's F12 mixture (DMEM/F12), 10% fetal bovine serum, 1% Penicillin/Streptomycin (Pen/Strep) and 1% GlutaMAX, dissociated in Hyaluronidase and Collagenase (all from Life Technologies) overnight and cultured for 2–4 weeks, as previously described (Dilwali et al., 2014). Human VS cell line HEI-193, derived from a patient with neurofibromatosis type 2 (NF2), was obtained from Dr. Giovanni at the House Ear Institute (Hung et al., 2002a,b).

#### 2.7. Pharmacologic treatment of VS cultures with BAY 11-7082, curcumin and siRNA

For siRNA treatment, cultured primary VS cells or HEI-193 cells were placed in antibiotic- and serum-free media overnight as instructed by manufacturer. The next day, the cells were incubated with siRNAs targeting NF- $\kappa$ B genes RELA (#s11915) and NFKB1 (#s9504), with control cells being treated with scrambled siRNA (#TR30015), for 5 days (all purchased

from Life Technologies). The vehicle used for siRNA delivery was RNAiMax (#13778030, Life Technologies). Some cultures were incubated with a fluorescent red oligo (Life Technologies) with vehicle to assess transfection efficiency.

For pharmacologic treatment, cultured primary VS cells, primary SCs and HEI-193 cells were treated for 48 h with NF- $\kappa$ B inhibitors BAY 11-7082 (BAY11, #sc-200615) or curcumin (#sc-200509A) (both purchased from Santa Cruz Biotechnology) in media fortified with antibiotics and serum. BAY11 or curcumin, diluted in 100% DMSO, were mixed to the accurate concentrations in media and applied to the cultures (with DMSO concentration in media being 1% maximum), alongside no treatment (NT) with DMSO alone.

#### 2.8. Quantification of proliferation and apoptosis

After treatment, proliferation or apoptosis was assessed as previously detailed (Dilwali et al., 2014). Briefly, cell proliferation was quantified by adding 5-Bromo-2'-Deoxyuridine (BrdU, Invitrogen) to the cultured cells 20 h prior to fixation. Primary antibodies against BrdU (AbD Serotec, #OBT0030G), S100 (Dako, #Z031129), or p50 (Abcam, #ab7971) were used. For assessing apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Roche Applied Sciences) was applied for 1 h at  $37^{\circ}\text{C}$  and 0.5 h at RT on the shaker after fixation and permeabilization. Cells were counted by an investigator (S.D.) blinded to the treatment conditions. Cells were counted in  $\geq 3$  fields. Cell proliferation and apoptosis were reported as percent BrdU positive and TUNEL positive nuclei, respectively. As a validation for TUNEL staining, apoptosis was also assessed using immunocytochemistry by the expression of cleaved caspase-3 in cells treated with siRNA or curcumin. Antibody against cleaved caspase-3 (Cell Signaling Technology, #9661) was utilized. The inhibitors were compared to the control group by normalizing the percent change in proliferation or percent of apoptosis in comparison to the non-treated cells.

#### 2.9. Electrophoretic mobility shift assay (gel shift assay)

The gel shift assay was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific, #20148) according to the manufacturer's manual. The 6% DNA retardation gels and biotin labeled NF- $\kappa$ B binding site oligos (sense: 5'-AGTT-GAGGGGACTTTCAGGC-biotin-3' and anti-sense: 5'-TCAACTCCCCTGAAAGGGTCCG-biotin-3'), and non-labeled oligos were purchased from Invitrogen. The nuclear extract of VS tumors and control GAN tissues were purified using the Nuclear Extraction Kit (Abcam, #ab113474).

#### 2.10. Statistical analyses

Networks from IPA were statistically analyzed with the right-tailed Fisher's exact test;  $p < 0.05$  was considered significant. For qPCR, western blot and treatment of cultured cells, two-tailed t-test was used to assess significance with a  $p < 0.05$  considered significant after a Benjamini-Hochberg correction for multiple hypotheses.



### 3. Results

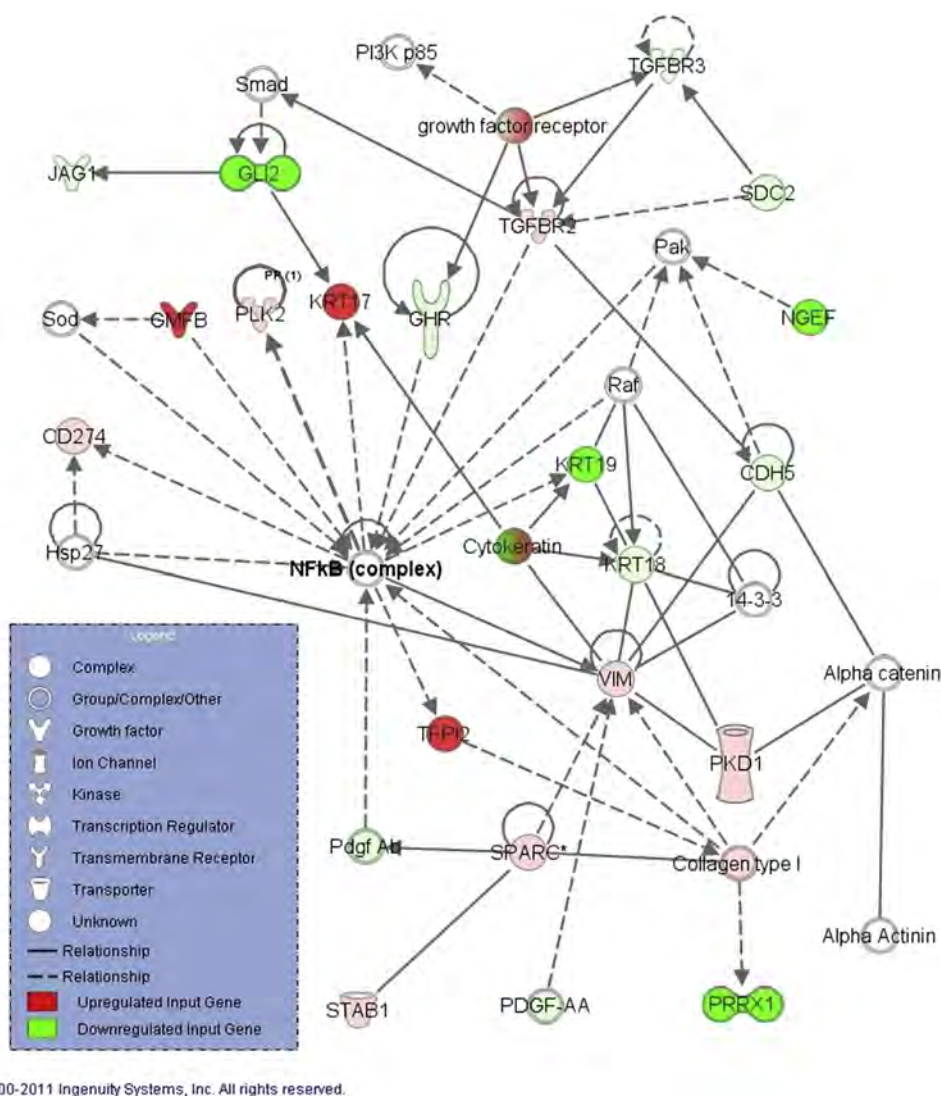
#### 3.1. Network analysis reveals nuclear factor-kappa B (NF-κB) as a central modulator of VS growth

Of the 622 articles identified, 19 met our inclusion criteria (Aarhus et al., 2010; Archibald et al., 2010; Bian et al., 2005; Cayé-Thomasen et al., 2010; Cioffi et al., 2010; Dayalan et al., 2006; Doherty et al., 2008; Kramer et al., 2010; Lassaletta et al., 2009; O'Reilly et al., 2004; Patel et al., 2008; Plotkin et al., 2009; Sawaya and Highsmith, 1988; Saydam et al., 2011; Seol et al., 2005; Stankovic et al., 2009; Szeremeta et al., 1995; Thomas et al., 2005; Welling et al., 2002), generating 221 molecules eligible for network analysis: 162 overexpressed and 59 underexpressed molecules in sporadic VS. IPA generated a total of 19 networks. [Supplementary Table S1](#) shows the hubs of the top 14 networks. Here we focus on validation

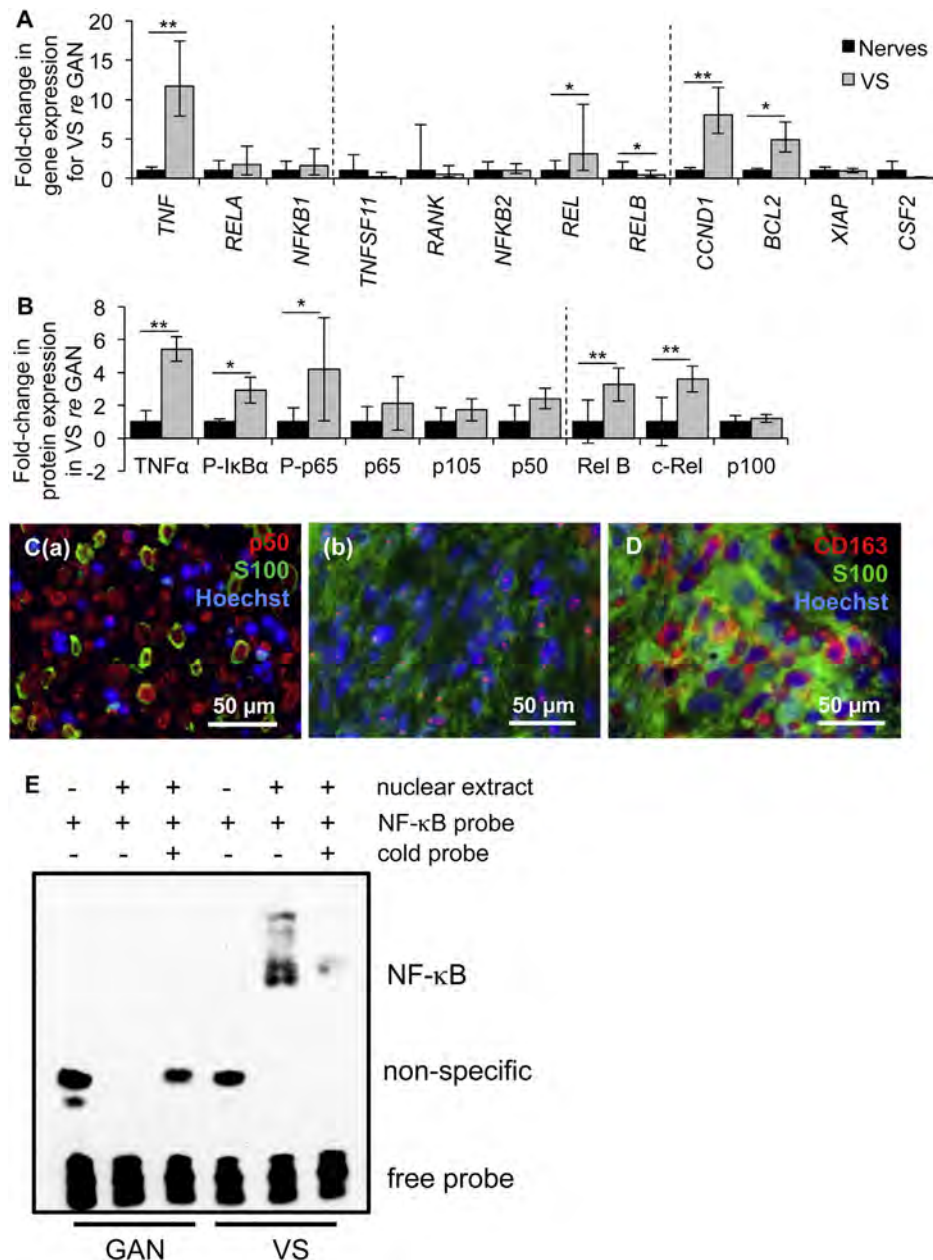
of the hub of the second most significant network ( $p = 10^{-33}$ , [Figure 1](#)). We focus on NF-κB because it is a key pro-inflammatory transcription factor that could be an important therapeutic target in VS ([Ammoun et al., 2013](#)), and TNFα, an inducer of NF-κB, was the hub of another top-ranking network ([Supplementary Table S1](#)).

#### 3.2. Vestibular schwannomas have aberrant expression and activation of the canonical NF-κB pathway

The canonical and non-canonical NF-κB pathways were investigated in VS compared to GAN using qPCR, western blot and immunohistochemistry. The qPCR data are expressed as the average with range of expression in parentheses and “n” indicating the number of different tumors or control nerves. In the canonical pathway, expression of genes NFKB1 (encoding the p50 subunit) and RELA (encoding the p65 subunit) tended to



**Figure 1** – A highly significant network ( $p = 10^{-33}$ ) that connects molecules reported to be upregulated (green) or downregulated (red) in VS with other molecules from IPA (white). The hub of this network is nuclear factor-kappa B (NF-κB) complex. Solid lines represent direct and dashed lines represent indirect interactions.



**Figure 2** – NF-κB is aberrantly activated in VS. **A**. NF-κB pathway expression in human VSs ( $n \geq 9$  tumors) versus GANs ( $n \geq 8$  nerves) as measured through qPCR. Dashed lines separate genes by groups, being genes associated with canonical NF-κB pathway, non-canonical NF-κB pathway and downstream targets of NF-κB. Error bars represent range. **B**. NF-κB pathway expression in human VSs ( $n \geq 4$ ) versus GANs ( $n \geq 4$ ) as quantified through western blot analysis. P-means phosphorylated protein. Dashed line separates canonical and non-canonical proteins. Error bars represent SD. **C**. Representative images of p50 expression (red), as visualized through immunohistochemistry, in (a) VS and (b) GAN specimens. Schwann or schwannoma cells are labeled with S100 (green). **D**. Representative image of CD163 expression (red), schwannoma cells (S100, green). GAN and VS expression is shown in black and gray bars, respectively (**A**, **B**). \* $p < 0.05$ , \*\* $p < 0.01$ . Nuclei are labeled with Hoechst (blue) in (**C**, **D**). **E**. Gel shift results show interaction between nuclear extracts of GAN tissue (pooled from 6 different patients, lanes 2–3) or VS tissue (pooled from 4 different patients, lanes 5–6) with the NF-κB binding site. The interaction could be disrupted in VS nuclear extracts by adding excessive unlabeled NF-κB binding site (lane 6).

be higher in VSs ( $n = 10$ ) compared to GANs ( $n = 10$ ), albeit not significantly, with  $p = 0.18$  and  $0.17$ , respectively (Figure 2A). Non-canonical components REL, RELB and NFKB2 exhibited different patterns of expression. REL was 3.1 (1.0–9.4) fold higher in VSs ( $n = 13$ ) than in GANs ( $n = 10$ ) ( $p = 0.01$ , Figure 2A). NFKB2 had the same average expression in VSs

as GANs ( $p = 0.22$ , Figure 2A). Interestingly, RELB was 0.4 (0.2–1.0) fold downregulated in VSs ( $n = 13$ ) compared to GANs (range 0.5–2.1,  $n = 10$ ) ( $p = 0.02$ , Figure 2A).

Exploring the downstream genes with κB binding sites by qPCR, two genes under canonical NF-κB control were significantly upregulated in VSs ( $n = 15$ ) relative to GANs ( $n = 15$ ):



pro-proliferative *CCND1* at 8.1 (5.7–11.5) fold ( $p = 0.0007$ ) and anti-apoptotic *BCL2* at 4.9 (3.3–7.1) fold ( $p = 0.02$ , Figure 2A). The ranges in GAN were 0.7–1.4 and 0.8–1.3 for *CCND1* and *BCL2*, respectively. Anti-apoptotic *XIAP* was equally expressed in VSs ( $n = 12$ ) and GANs ( $n = 7$ ) ( $p = 0.18$ , Figure 2A). Pro-proliferative *CSF2* tended to be downregulated, albeit not significantly ( $p = 0.11$ , Figure 2A), in VSs ( $n = 9$ ) compared to GANs ( $n = 7$ ).

Upstream regulator of the canonical NF- $\kappa$ B pathway, gene *TNF* encoding *TNF $\alpha$* , was expressed at 11.7 (7.9–17.4) fold higher levels in VSs ( $n = 10$ ) than in GANs (range 0.7–1.5,  $n = 10$ ) ( $p = 0.003$ , Figure 2A). Upstream regulator of the non-canonical NF- $\kappa$ B pathway, *RANKL* gene *TNFSF11* tended to be downregulated in VSs ( $n = 10$ ) than in GANs ( $n = 10$ ), although not significantly ( $p = 0.20$ , Figure 2A).

Following qPCR, NF- $\kappa$ B translation and activation were assessed. Western blot analysis revealed that NF- $\kappa$ B canonical pathway was substantially activated in VSs compared to GANs. *TNF $\alpha$*  activates inhibitor of kappa B kinase (*I $\kappa$ B $\kappa$* ), which phosphorylates inhibitor of kappa B alpha (*I $\kappa$ B $\alpha$* ), enabling the heterodimer of NF- $\kappa$ B p65 and p50 to phosphorylate and relocate to the nucleus to promote transcription of genes important for survival and proliferation (Karin, 1999). Western blot data are summarized as average fold change  $\pm$  standard deviation, with “n” indicating the number of different tumors or nerves. The representative western blot results are shown in Supplementary Figure S1 and the statistical results are shown in Figure 2B. The internal control protein, GAPDH, was not significantly different between VSs and GANs ( $p = 0.36$ ). NF- $\kappa$ B p65 (encoded by the *RELA* gene) and p105 (encoded by the *NFKB1* gene) had an insignificant trend of being more abundant in VSs ( $n = 7$ –10) than in GANs ( $n = 7$ –9), with  $p = 0.09$  and  $p = 0.14$ , respectively (Figure 2B). The phosphorylated form of p65 was  $4.2 \pm 3.1$  fold more expressed in VSs ( $n = 9$ ) compared to GANs ( $n = 8$ ,  $p = 0.03$ , Figure 2B). p105's derived subunit p50 likewise showed tendency for higher expression, albeit non-significant, in VSs ( $n = 15$ ) compared to GANs ( $n = 11$ ,  $p = 0.10$ , Figure 2B). NF- $\kappa$ B's canonical inducer, *TNF $\alpha$* , was  $5.4 \pm 0.7$  fold more abundant in VS ( $n = 4$ ) than in GAN ( $n = 4$ ,  $p = 0.001$ , Figure 2B), demonstrating the same trend as seen through qPCR. The phosphorylated form of *I $\kappa$ B $\alpha$*  was also  $2.8 \pm 0.8$  fold higher in VSs ( $n = 4$ ) than in GANs ( $n = 4$ ,  $p = 0.01$ , Figure 2B).

The expression of NF- $\kappa$ B non-canonical proteins c-Rel (encoded by *REL* gene) and p100 (encoded by *NFKB2* gene) mirrored the corresponding mRNA expression: c-Rel was  $3.6 \pm 0.8$  fold more expressed in VSs ( $n = 7$ ) than in GANs ( $n = 7$ ,  $p = 0.003$ , Figure 2B). p100 was not different in VSs ( $n = 4$ ) compared to GANs ( $n = 4$ ,  $p = 0.42$ , Figure 2B). Interestingly, expression of Rel B protein (encoded by *RELB*) was significantly,  $3.3 \pm 1$  fold higher in VSs ( $n = 7$ ) than in GANs ( $n = 7$ ,  $p = 0.006$ ), demonstrating the opposite trend from the corresponding mRNA. Taken together, these results demonstrate presence and basal activation of NF- $\kappa$ B in GANs and VSs, and consistently higher activation of the canonical NF- $\kappa$ B pathway in VSs.

Immunohistochemistry in 5 different VS and 4 different GAN samples verified that NF- $\kappa$ B was active in VS as the p50 subunit localized to the nuclei in VS specimens (Figure 2C (b)), thus corroborating the western blot results

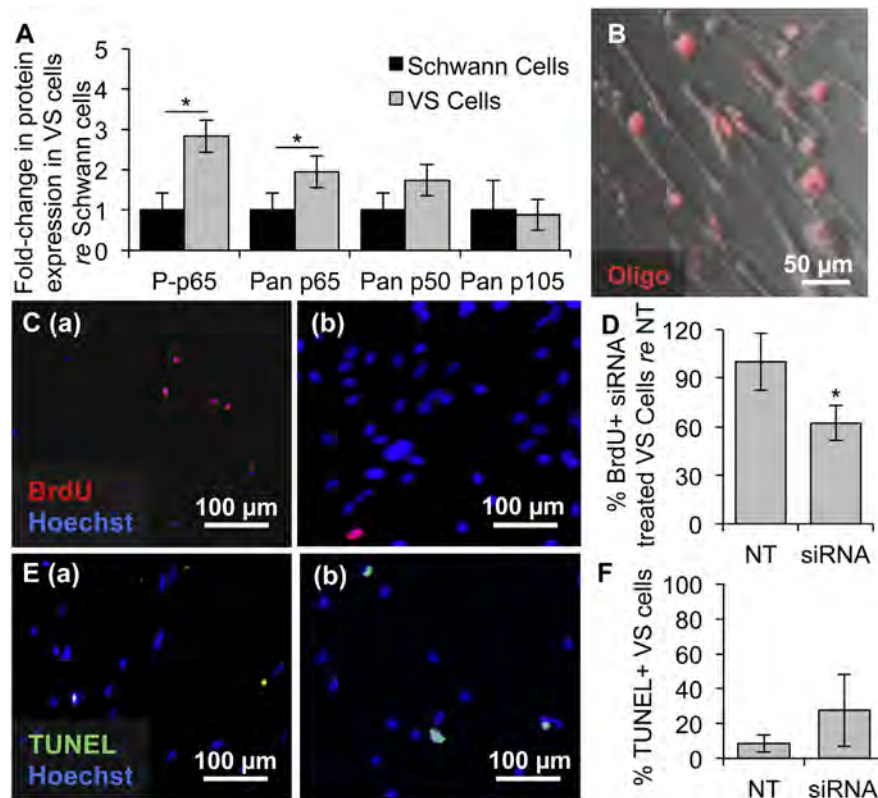
demonstrating a higher level of phosphorylation, and hence activation of NF- $\kappa$ B in VSs. GAN specimens showed minimal p50 nuclear localization although p50 was present in the cytoplasm (Figure 2C (a)). S100, a marker for SCs, highlights schwannoma cells in VS specimens and SCs encasing the nerve fibrils in GANs (Figure 2C (a)). Additionally, CD163-positive tumor-associated macrophages were present in the same VSs at substantially higher levels than in GANs (Figure 2D), indicating an aberrant inflammatory presence in VS as described previously (de Vries et al., 2013). Taken together, our results demonstrating activation of p50 and several other proteins associated with NF- $\kappa$ B in VS expand on the previous finding of p65 activation in VS cells (Ammoun et al., 2013).

To further confirm the activation of the NF- $\kappa$ B pathway in VS tissues, the gel shift assay was performed and the DNA binding activities in VS tissues and GAN tissues were compared. To provide enough nuclear extracts for the assay, VS tissues were pooled from 4 patients and GAN tissues were pooled from 6 patients. The binding of NF- $\kappa$ B p65-p50 heterodimer to its binding site was avid in the VS nuclear extract and not detectable in the GAN nuclear extract (Figure 2E). The interaction in the VS nuclear extract could be blocked by adding the excess non-labeled oligos containing the NF- $\kappa$ B binding site (Figure 2E, lane 6). These results indicate that the NF- $\kappa$ B activity was specifically elevated in VS tissues.

### 3.3. Specific NF- $\kappa$ B knockdown decreases proliferation and survival of VS cultured cells

The NF- $\kappa$ B canonical pathway was also expressed and activated at significantly higher levels in primary VS cultures ( $n = 6$  different tumors) compared to SC cultures ( $n = 6$  different nerves) (Figure 3A). NF- $\kappa$ B p65 and its phosphorylated form had  $1.9 \pm 0.4$  fold ( $p = 0.01$ ) and  $2.8 \pm 0.4$  fold ( $p = 0.02$ ) higher expression in VS cells compared to SCs, respectively. NF- $\kappa$ B p105 and its derived subunit p50 were present in cultures, although not at significantly higher levels than in SCs ( $p = 1.0$  and  $p = 0.06$ , respectively, Figure 3A).

Applying siRNAs targeting *RELA* and *NFKB1* concurrently decreased proliferation, as measured by nuclear BrdU staining, and cell survival, as measured by the TUNEL assay. Results are summarized as average  $\pm$  standard error of mean (SEM), with “n” referring to the number of cultures from different specimens. Proliferation changes are normalized to each culture's proliferation rate. Transfection efficiency of approximately  $94 \pm 3\%$  was achieved in primary VS cells ( $n = 3$ ), as assessed by transfection of a fluorescent red-labeled oligo (Figure 3B). The siRNA-mediated knockdown of *NFKB1* and *RELA* in VS cells was detected using western blot and the results are shown in Supplementary Figures S2A and S2B, respectively. Basal proliferation in VS cultures treated with scrambled siRNA was  $6.5\% \pm 2.6\%$  ( $n = 4$ , Figure 3C (a), D). Proliferation significantly decreased to  $62.33\% \pm 10.59\%$  after siRNA treatment ( $n = 4$ ,  $p = 0.025$ , Figure 3C (b), D). Percentage of VS cells treated with scrambled siRNA only exhibiting TUNEL staining was  $8.59\% \pm 4.92\%$  ( $n = 3$ , Figure 3E (a), F). Apoptosis tended to increase, although insignificantly, to  $27.54\% \pm 20.53\%$  in VS cultures treated with



**Figure 3** – NF- $\kappa$ B is aberrantly activated in primary VS cultures and its siRNA-mediated knockdown decreases proliferation. **A.** NF- $\kappa$ B expression in cultured human VSs ( $n \geq 6$  tumors) normalized to expression in SC cultures ( $n \geq 6$  nerves) as quantified through western blot analysis. P-means phosphorylated protein. Error bars represent SD. **B.** Representative image of effective transfection of a fluorescently labeled oligonucleotide (oligo, red) in primary VS cells. **C.** Representative proliferation images are shown for (a) scrambled siRNA or (b) siRNA treated primary VS cells. BrdU in nuclei (red) marks proliferating cells. **D.** Quantification of proliferation changes after siRNA treatment in primary VS cells normalized to proliferation in control scrambled siRNA treated (NT) cells ( $n = 4$  results were from 4 independent results from cultures of two different patients). **E.** Representative cell death images are shown for (a) scrambled siRNA and (b) siRNA treated primary VS cells. TUNEL (green) in nuclei marks dying cells. **F.** Quantification of cell death rate after siRNA treatment of primary VS cells as measured by TUNEL staining ( $n = 3$  different cultures). Error bars represent SD for panels D and F. \* $p = 0.025$ , re = compared to. Nuclei are labeled with Hoechst (blue) in (C, E).

NF- $\kappa$ B siRNA ( $n = 3$ ,  $p = 0.53$ , [Figure 3E](#) (b), F). Similar results were also observed using anti-cleaved caspase-3 immunocytochemistry. NF- $\kappa$ B siRNA transfection in VS increased the percentage of cells that expressed cleaved caspase-3 from  $2.01\% \pm 1.24\%$  to  $7.44\% \pm 7.15\%$ ; however, the difference was not statistically significant ( $n = 3$ ,  $p = 0.13$ , [Fig S3A](#)).

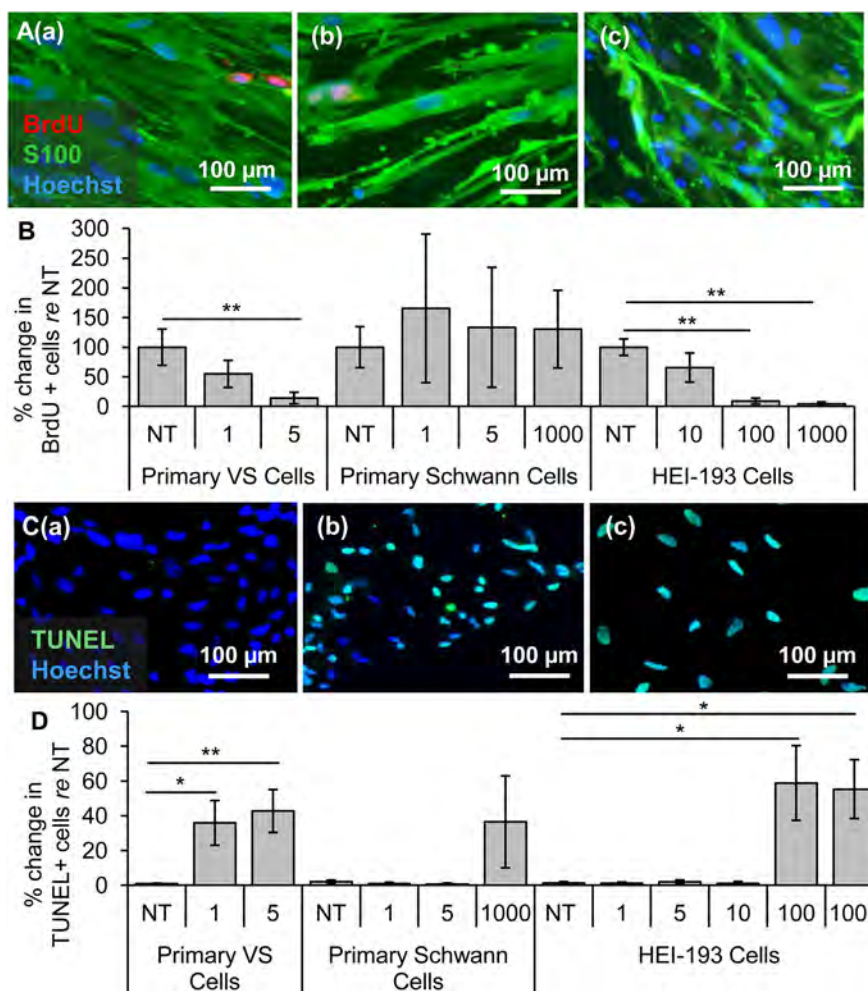
### 3.4. NF- $\kappa$ B small-molecule inhibitor BAY 11-7082 decreases proliferation and survival selectively in primary VS and HEI-193 cells

Primary VS cells, control SC cultures and the HEI-193 cell line were treated with BAY11. BAY11 treatment significantly decreased the activity of the NF- $\kappa$ B pathway as shown by the decrease of phosphorylated p65 in western blot ([Fig. S2C](#), left). Results are reported using the same format and meaning of “n” and “p” as for siRNA application. Treatment with 1 and 5  $\mu$ M BAY11 changed proliferation in VS cells to  $54.7 \pm 22.8\%$  ( $n = 5$ ,  $p = 0.15$ , [Figure 4A](#) (b), B) and  $14.3 \pm 9.7\%$  ( $n = 4$ ,  $p = 0.002$ , [Figure 4A](#) (c), B) of the non-treated cells (NT, [Figure 4A](#) (a)), respectively. The apoptotic rate changed from

$1.1 \pm 0.27\%$  ([Figure 4C](#) (a), D) in the NT VS cells to  $36 \pm 13\%$  ( $n = 7$ ,  $p = 0.06$ , [Figure 4C](#) (b), D) and  $47 \pm 12\%$  ( $n = 8$ ,  $p = 0.02$ , [Figure 4C\(c\)](#), D) in cells treated with 1  $\mu$ M and 5  $\mu$ M BAY11, respectively.

In the control SC cultures, normalized proliferation rates did not change significantly, being  $100.0 \pm 34.7\%$ ,  $165.2 \pm 125.1\%$  ( $p = 0.70$ ),  $133.2 \pm 101.1\%$  ( $p = 0.69$ ),  $130.2 \pm 65.6\%$  ( $p = 0.78$ ) for NT cells, 1  $\mu$ M, 5  $\mu$ M and 1 mM BAY11, respectively ( $n = 3$ , [Figure 4B](#)). SCs demonstrated higher apoptosis only at the highest, 1 mM BAY11 treatment. NT, 1  $\mu$ M, 5  $\mu$ M or 1 mM treated GAN cells exhibited apoptosis rates of  $2.0 \pm 0.9\%$ ,  $1.0 \pm 0.7\%$  ( $p = 0.53$ ),  $0.7 \pm 0.7\%$  ( $p = 0.47$ ) and  $36.5 \pm 26.5\%$  ( $p = 0.43$ ), respectively ( $n = 3$ , [Figure 4D](#)). These control experiments suggest that 5  $\mu$ M BAY11 has the greatest therapeutic promise against VS without being toxic to SCs.

BAY11 treatment also decreased HEI-193 cell survival in a dose-dependent manner. HEI-193 cells had very high basal proliferation rates of  $84.9 \pm 11.7\%$  ( $n = 3$ ). NT, 10  $\mu$ M, 100  $\mu$ M and 1 mM BAY11 treated HEI-193 cells exhibited normalized proliferation rates of  $100.0 \pm 13.8\%$ ,  $65.6 \pm 24.6\%$  ( $n = 5$ ,



**Figure 4** – NF- $\kappa$ B inhibitor BAY11-7082 leads to selective decrease in proliferation and survival of VS cells. **A.** Representative proliferation images for primary VS cultures treated with (a) no treatment (NT), (b) 1  $\mu$ M and (c) 5  $\mu$ M BAY11-7082 (BAY11). BrdU in nuclei (red) marks proliferating cells, S100 (green) marks schwannoma cells. **B.** Quantification of proliferation changes after treatment with BAY11 at different concentrations (given in  $\mu$ M) in primary VS cells, primary SCs and HEI-193 NF2 VS cell line, all normalized to proliferation in control NT cells ( $n \geq 3$ ). **C.** Representative cell death images are shown for primary VS cultures treated with (a) NT, (b) 1  $\mu$ M and (c) 5  $\mu$ M BAY11-7082 (BAY11). TUNEL (green) in nuclei marks dying cells. **D.** Quantification of cell death rate after treatment with BAY11 at different concentrations (given in  $\mu$ M) in primary VS cells, primary SCs and HEI-193 NF2 VS cell line ( $n \geq 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , re = compared to. Error bars represent SEM. Nuclei are labeled with Hoechst (blue) in (A, C).

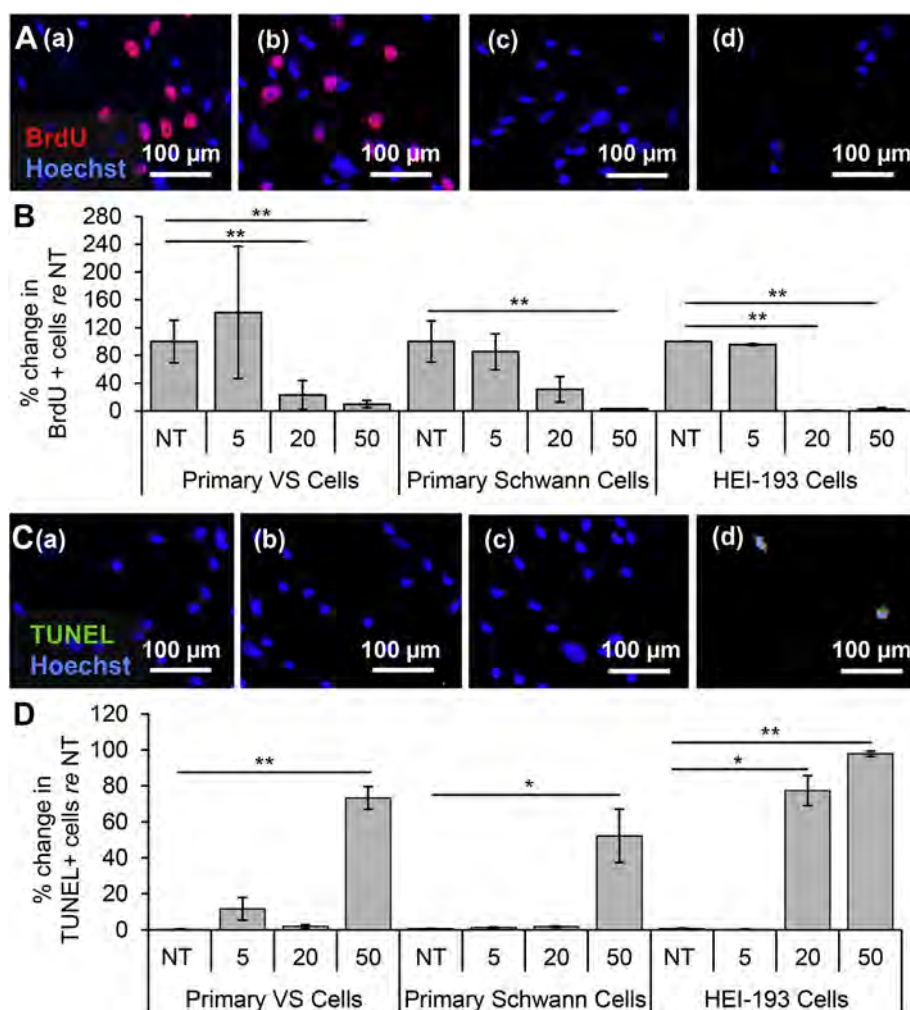
$p = 0.25$ ),  $9.1 \pm 4.9\%$  ( $n = 5$ ,  $p = 0.006$ ) and  $4.3 \pm 3.3\%$  ( $n = 5$ ,  $p = 0.003$ ), respectively (Figure 4B). NT, 1, 5, 10, 100  $\mu$ M and 1 mM BAY11 treated HEI-193 cells exhibited apoptotic rates of  $1.3 \pm 0.8\%$ ,  $1.3 \pm 0.5\%$  ( $n = 6$ ,  $p = 0.22$ ),  $1.9 \pm 1.2\%$  ( $n = 6$ ,  $p = 0.26$ ),  $1.1 \pm 1.1\%$  ( $n = 5$ ,  $p = 0.63$ ),  $58.8 \pm 21.5\%$  ( $n = 5$ ,  $p = 0.04$ ) and  $55.3 \pm 16.9\%$  ( $n = 5$ ,  $p = 0.02$ ), respectively (Figure 4D).

### 3.5. Clinically-relevant NF- $\kappa$ B inhibitor curcumin decreases proliferation and survival in cultured primary VS cells, NF2 VS cell line and primary SCs

Curcumin, a natural, well-tolerated NF- $\kappa$ B inhibitor that is currently used in many clinical trials for various neurological, inflammatory and neoplastic diseases, ranging from Alzheimer's disease to colon cancer (Hatcher et al., 2008), was

tested in VS cells. Treatment of curcumin significantly decreased the activity of the NF- $\kappa$ B pathway as shown by the decrease of phosphorylated p65 in western blot (Fig. S2, right). Results are reported using the same format and meaning of "n" and "p" as for siRNA application. Proliferation decreased in a dose-dependent manner in VS cultures, with VS cells receiving NT, 5, 20 and 50  $\mu$ M curcumin (Figure 5A (a–d, respectively)) exhibiting normalized proliferation rates of  $100.0\% \pm 30.5\%$ ,  $141.8\% \pm 95.2\%$  ( $p = 0.57$ ),  $23.0 \pm 20.9\%$  ( $p = 0.03$ ) and  $9.8 \pm 5.3$  ( $p = 0.0005$ ) ( $n = 3$ , Figure 5B). Apoptosis also increased in a dose-dependent manner, with VS cells receiving NT, 5, 20 or 50  $\mu$ M curcumin (Figure 5C (a–d, respectively)) exhibiting apoptotic rates of  $0.3 \pm 0.1$ ,  $11.6 \pm 6.3\%$  ( $n = 8$ ,  $p = 0.37$ ),  $1.8 \pm 1.0\%$  ( $n = 3$ ,  $p = 0.37$ ) and  $73.3 \pm 6.3\%$  ( $n = 7$ ,  $p = 0.0005$ ) (Figure 5D). The effect of curcumin treatment on apoptosis in VS cells was also investigated by





**Figure 5 – Clinically-relevant NF- $\kappa$ B inhibitor curcumin leads to selective decrease in proliferation and survival of VS cells.** A. Representative proliferation images for primary VS cultures treated with (a) no treatment (NT), (b) 5, (c) 20, and (d) 50  $\mu$ M curcumin. BrdU in nuclei (red) marks proliferating cells. B. Quantification of proliferation changes after treatment with curcumin at 5, 20, and 50  $\mu$ M in primary VS cells, primary Schwann cells and HEI-193 NF22 VS cell line, all normalized to proliferation in control NT cells ( $n \geq 3$ ); C. Representative cell death images are shown for primary VS cultures treated with (a) NT, (b) 5, (c) 20, and (d) 50  $\mu$ M curcumin. TUNEL (green) marks dying cells. D. Quantification of cell death rate after treatment with curcumin at 5, 20, and 50  $\mu$ M in primary VS cells, primary Schwann cells and HEI-193 NF22 VS cell line ( $n \geq 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , re = compared to. Error bars represent SEM. Nuclei are labeled with Hoechst (blue) in (A, C).

cleaved caspase-3 immunocytochemistry. The results demonstrate a statistically significant apoptosis-inducing effect of 50  $\mu$ M curcumin in VS cells ( $n = 3$ ,  $p = 0.02$ , Fig. S3B). Taken together, these results suggest that the decrease of NF- $\kappa$ B by curcumin may be the mechanism leading to apoptosis in VS cells.

Surprisingly, in contrast to the seemingly well-tolerated profile for curcumin in humans, curcumin decreased proliferation and increased apoptosis in control SC cultures at concentrations comparable to those efficacious in VS cultures. Proliferation tended to decrease in a dose-dependent manner, with SCs receiving NT, 5, 20, 50  $\mu$ M curcumin exhibiting normalized proliferation rates of  $100.0\% \pm 29.6\%$ ,  $85.3 \pm 25.7\%$  ( $n = 4$ ,  $p = 0.33$ ),  $31.0 \pm 18.3\%$  ( $n = 4$ ,  $p = 0.13$ ) and  $3.14\%$  ( $n = 1$ ,  $p = 0.04$ ) (Figure 5B); the trend became significant only at the highest tested dose. Apoptosis had the same

trend with the highest dose leading to a significant increase in cell death. NT, 5, 20 or 50  $\mu$ M treated GAN cells exhibited apoptotic rates of  $0.6 \pm 0.2$ ,  $1.3 \pm 0.3\%$  ( $n = 4$ ,  $p = 0.16$ ),  $1.7 \pm 0.4\%$  ( $n = 4$ ,  $p = 0.31$ ) and  $52.2 \pm 14.9\%$  ( $n = 5$ ,  $p = 0.03$ ) (Figure 5D). Nonetheless, the doses up to 20  $\mu$ M seemed selectively cytostatic against primary VS cells.

Intriguingly, the HEI-193 cells were more susceptible to curcumin than primary VS cells or healthy SCs. Proliferation decreased drastically with dose increases: HEI-193 cells receiving NT, 5, 20 or 50  $\mu$ M curcumin exhibiting normalized proliferation rates of  $100.0\% \pm 0.2\%$ ,  $95.0 \pm 1.6\%$  ( $p = 0.12$ ),  $0.4 \pm 0.4\%$  ( $p = 0.0001$ ) and  $2.3 \pm 2.3\%$  ( $p = 0.001$ ) ( $n = 3$ , Figure 5B). Apoptosis increased drastically at 20  $\mu$ M, in contrast to the primary VS cells exhibiting apoptosis at 50  $\mu$ M. HEI-193 cells receiving NT, 5, 20 or 50  $\mu$ M curcumin exhibited apoptotic rates of  $0.5 \pm 0.4$ ,  $0.3 \pm 0.1\%$  ( $n = 5$ ,

$p = 0.32$ ),  $77.3 \pm 8.4\%$  ( $n = 3$ ,  $p = 0.02$ ) and  $97.8 \pm 1.5\%$  ( $n = 4$ ,  $p = 0.00003$ ) (Figure 5D).

#### 4. Discussion

Conducting the first comprehensive network analysis of molecules implicated in VS pathobiology, we identified and validated NF- $\kappa$ B as a central regulator. We also found direct interactors of NF- $\kappa$ B such as PDGF (Olson et al., 2007), which have been implicated in VS progression, to be the hubs of other significant networks (Supplementary Table S1). Although others have suggested that NF- $\kappa$ B is activated in VS cells via upstream stimulation such as with p75 signaling (Ahmad et al., 2014), we find that NF- $\kappa$ B is inherently highly active in human VS tissue and derived primary VS cells. The apparent disparity may be due to differences in detection methods or sample processing. As all prior experiments had been conducted on cultured cells and cell lines, we are the first to show that the aberrant NF- $\kappa$ B activation occurs also in the freshly resected VS tissue and cannot be deemed an artifact of culturing.

Our analysis of expression signatures of the downstream NF- $\kappa$ B genes in VS suggests a unique NF- $\kappa$ B target gene program in VS, as may be expected in pathologic inflammation (Hoesel and Schmid, 2013). Since NF- $\kappa$ B is highly expressed by immature SCs during development, progressively declining from pre-myelinating SCs to near absence in mature myelinating SCs (Nickols et al., 2003), our findings are also consistent with VSs exhibiting a gene expression profile akin to immature SCs (Hung et al., 2002a,b). Pre-existing upregulation of NF- $\kappa$ B in VSs, along with a few defining mutations in other genes, could enable neoplastic proliferation of non-myelinating SCs.

Using a pre-clinical model of primary human VS cells, we demonstrate potential therapeutic efficacy of directly targeting NF- $\kappa$ B via experimental and clinical inhibitors. Our work with freshly harvested VS samples from different patients captures the variability of NF- $\kappa$ B aberrance in different VSs. Our results suggest that therapeutic targeting of NF- $\kappa$ B may be generally effective against VSs, not only against a small subset of VSs. By utilizing three different modalities to inhibit NF- $\kappa$ B: (1) highly-specific siRNAs against the NF- $\kappa$ B p50 and p65, (2) a pharmacologic inhibitor BAY11 and (3) a clinically-relevant, natural inhibitor curcumin, we affirm NF- $\kappa$ B's role in VS proliferation and survival. We reinforce previous findings that siRNA mediated NF- $\kappa$ B knockdown in primary VS cells reduces proliferation and survival (Ammoun et al., 2013), and expand on them by using more clinically relevant inhibitors.

A small molecule NF- $\kappa$ B inhibitor BAY11 showed a high level of efficacy and specificity against VS cells. Although BAY11 has been characterized as an effective inhibitor of NF- $\kappa$ B by inhibiting I $\kappa$ K activation, recently BAY11 has been recognized to target many other pro-inflammatory molecules, including TNF $\alpha$  (Lee et al., 2012a,b). Future work is needed to determine whether the therapeutic efficacy of BAY11 against VS cells is solely due to NF- $\kappa$ B inhibition. As BAY11 was not cytotoxic in primary SCs and has been well tolerated in vivo in murine tumor xenograft studies (Dewan et al., 2003), future

exploration of BAY11 against VS in animal models in vivo is warranted.

Curcumin, a clinically relevant NF- $\kappa$ B inhibitor that has been tested in many clinical trials (Hatcher et al., 2008), inhibited proliferation and promoted apoptosis of both primary VS cells and HEI-193 VS cells. Curcumin's greater effectiveness against HEI-193 VS cells at a lower dose than required for primary VS cells suggests a higher therapeutic efficacy against NF2-derived than sporadic VSs. The dosage curve of curcumin resembles a previously established dosage curve for HEI-193 cells in a study that focused on another molecule through which curcumin may be acting: Hsp70 (Angelo et al., 2011). A follow-up study by the same authors investigating curcumin's direct binding partners did not reveal the NF- $\kappa$ B complex being a target in HEI-193 cells (Angelo et al., 2013), although the authors had previously reported inhibition of phosphorylation of Protein Kinase B (AKT), an upstream regulator of NF- $\kappa$ B activation (Angelo et al., 2011; Bai et al., 2009). This is in contrast to the large body of literature that shows curcumin's role as an NF- $\kappa$ B inhibitor, via inhibition of TNF $\alpha$ -induced I $\kappa$ B degradation (Hatcher et al., 2008), and as a general inhibitor of inflammation (Hatcher et al., 2008; Marin et al., 2007). It is important to acknowledge that although curcumin was found to be efficacious against colon cancer and Alzheimer's disease in animal and human studies, therapeutic and toxicity profiles of curcumin have not been comprehensively elucidated (Burgos-Morón et al., 2010). Some clinical trials have noted nausea and diarrhea in patients taking curcumin (Burgos-Morón et al., 2010). Since the levels of curcumin that led to primary VS and SC death were comparable, more research is required on curcumin's toxicity profile, best formulation and administration methods and its efficacy in brain diseases. Importantly, however, curcumin has recently been shown to have otoprotective effect against aminoglycoside toxicity and the associated hearing loss (HL) (Salehi et al., 2014). Since most VS patients present with HL, future studies are needed to explore whether curcumin could attenuate both VS growth and the associated HL simultaneously.

By establishing aberrance of several molecules involved in the NF- $\kappa$ B pathway and efficacy of NF- $\kappa$ B inhibition selectively in VS cells via several inhibitors, we demonstrate NF- $\kappa$ B as a potential pharmacologic target against VS. However, possible future clinical targeting of NF- $\kappa$ B has to be considered carefully given that NF- $\kappa$ B is an important signaling node that most cells rely on.

#### Conflicts of interest

All authors have no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molonc.2015.03.009>.

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## ORIGINAL ARTICLE

## Nonsteroidal anti-inflammatory medications are cytostatic against human vestibular schwannomas

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Vestibular schwannomas (VSs) are the most common tumors of the cerebellopontine angle. Significant clinical need exists for pharmacotherapies against VSs. Motivated by previous findings that immunohistochemical expression of cyclooxygenase 2 (COX-2) correlates with VS growth rate, we investigated the role of COX-2 in VSs and tested COX-2 inhibiting salicylates against VSs. COX-2 was found to be aberrantly expressed in human VS and primary human VS cells in comparison with control human nerve specimens and primary Schwann cells (SCs), respectively. Furthermore, levels of prostaglandin E2, the downstream enzymatic product of COX-2, were correlated with primary VS culture proliferation rate. Because COX-2 inhibiting salicylates such as aspirin are well tolerated and frequently clinically used, we assessed their repurposing for VS. Changes in proliferation, cell death, and cell viability were analyzed in primary VS cultures treated with aspirin, sodium salicylate, or 5-aminosalicylic acid. These drugs neither increased VS cell death nor affected healthy SCs. The cytostatic effect of aspirin in vitro was in concurrence with our previous clinical finding that patients with VS taking aspirin demonstrate reduced tumor growth. Overall, this work suggests that COX-2 is a key modulator in VS cell proliferation and survival and highlights salicylates as promising pharmacotherapies against VS. (Translational Research 2015; ■:1–11)

**Abbreviations:** 5-ASA = 5-aminosalicylic acid; BrdU = 5-bromo-2'-deoxyuridine; GAN = great auricular nerve; COX-2 = cyclooxygenase 2; I $\kappa$ B = I kappa B kinase; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaSal = sodium salicylate; NF- $\kappa$ B = nuclear factor kappa-light-chain-enhancer of activated B; PBS = phosphate-buffered saline; PBST = phosphate-buffered saline with Tween 20; PTG = prostaglandin; *PTGS2* = gene encoding COX-2 protein; RIPA = Radioimmunoprecipitation Assay; S100 = Schwann cell/schwannoma cell marker; SC = Schwann cell; SD = standard deviation; SEM = standard error of the mean; VS = vestibular schwannoma

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**AT A GLANCE COMMENTARY****Dilwali S, et al.****Background**

Vestibular schwannomas (VSs) are the most common tumors of the cerebellopontine angle. Significant clinical need exists for pharmacotherapies against VSs.

**Translational Significance**

Cyclooxygenase 2 was found to be aberrantly expressed and active in human VSs. Well-tolerated and clinically relevant salicylates, namely aspirin, sodium salicylate, and 5-aminosalicylic acid, significantly reduced proliferation in primary human VS cultures. This work suggests that cyclooxygenase 2 is a key modulator in VS cell proliferation and survival and highlights salicylates as promising pharmacotherapies against VS.

**INTRODUCTION**

Vestibular schwannomas (VSs) are the most common tumors of the cerebellopontine angle and the fourth most common intracranial tumors.<sup>1</sup> Although VSs are histologically nonmalignant, they can lead to substantial morbidity, including sensorineural hearing loss, vestibular dysfunction, and facial nerve paralysis, because of their location within the internal auditory canal and the cerebellopontine angle.<sup>1,2</sup> Large VSs can cause additional paralysis of other cranial nerves, brainstem compression, and death.<sup>2</sup> Currently, patients with symptomatic or growing VSs can undergo surgical resection or radiotherapy. Both these procedures can result in serious complications. Surgical resection entails full or partial removal of the tumor via craniotomy and carries substantial risks, including sensorineural hearing loss, vestibular dysfunction, facial nerve paralysis, cerebrospinal fluid leaks, and meningitis.<sup>3,4</sup> Stereotactic radiotherapy entails delivering a radiation dose to the tumor and can be associated with severe adverse effects such as further exacerbation of the sensorineural hearing loss, vestibular dysfunction, and potential malignant transformation of the tumor.<sup>5,6</sup> Patients with nongrowing or asymptomatic VSs can undergo conservative management and follow tumor progression through serial magnetic resonance imaging, but because of the lack of biomarkers for VS growth and associated symptoms, conservative monitoring can be a risky approach.<sup>7</sup> Effective drug

therapies that can limit VS growth would greatly advance health care for patients with VS.

Cyclooxygenase 2 (COX-2), a major inflammatory mediator, has been implicated in VS. Previous studies demonstrate that the expression level of COX-2 in VSs is correlated with tumor proliferation rates, as judged by the intensity of COX-2 immunostaining in VS specimens.<sup>8</sup> The COX enzymes catalyze the biosynthesis of prostaglandins (PTGs), hormone-like lipid compounds that can trigger the inflammatory response.<sup>9</sup> In contrast to COX-1, which is expressed constitutively as a homeostatic enzyme in several cell types such as platelets and gastrointestinal mucosal cells, COX-2 is expressed at sites of inflammation and neoplasia.<sup>8,9</sup> Specifically, COX-2 has been described to modulate cell proliferation and apoptosis in many solid tumors, such as colorectal, breast, and prostate cancers.<sup>9</sup>

Salicylates, a class of nonsteroidal anti-inflammatory drugs (NSAIDs) defined by their chemical structure, are attractive therapeutics because they are clinically relevant, well-tolerated, effective COX-2 inhibitors, commonly used against pathologies such as pain and arthritis.<sup>10</sup> Furthermore, in some cases, chronic intake of salicylates has led to a significant reduction in the incidence and burden of various tumors, such as colorectal cancer.<sup>9</sup> In our study, we assessed the efficacy of 3 different salicylates, aspirin, sodium salicylate (NaSal), and 5-aminosalicylic acid (5-ASA), against VS because they are clinically used and well tolerated. Specifically, aspirin has been confirmed to provide chemoprevention for multiple human malignancies, including colon, gastric, breast, and prostate cancer—reviewed in Thorat and Cuzick.<sup>11</sup> NaSal is a sodium salt of salicylic acid. It is used clinically as an analgesic and antipyretic and as an alternative to aspirin for people sensitive to aspirin. NaSal has shown effectiveness against myeloid leukemia cell lines.<sup>12</sup> 5-ASA is commonly used to treat inflammatory bowel disease including ulcerative colitis<sup>13</sup> and Crohn's disease,<sup>14</sup> and it can prevent colorectal cancer.<sup>15</sup> In addition to its anti-inflammatory properties, 5-ASA is thought to be an antioxidant that traps free radicals.<sup>16</sup> These 3 salicylates, although acting through similar mechanisms to inhibit COX activity, have nuances that can lead to differential therapeutic and toxic profiles.<sup>10</sup> We explored the expression of COX-2 in human VS and the therapeutic efficacy of salicylate-mediated COX-2 inhibition in primary VS cells. All salicylates tested were effective in selectively reducing proliferation and viability of cultured VS cells, accompanied by reduced secreted PTG levels. Our work suggests

promising potential of commonly used salicylates against VS.

## MATERIALS AND METHODS

**Specimen collection.** Human great auricular nerves (GANs) were used as healthy control nerves and as the source for healthy human Schwann cells (SCs), as these nerves are routinely sacrificed for access during parotidectomies and neck dissections. Immediately after GAN resection, nerve specimens measuring 1 cm (from parotidectomies) to 5 cm (from neck dissections) were placed in sterile saline on ice and transported to the laboratory. Human VS tumor specimens were also collected from independent surgical resections via indicated craniotomies. Specimens were handled according to the institutional review board's study protocol approved by the Human Studies Committee of Massachusetts General Hospital and Massachusetts Eye and Ear Infirmary.

**Reverse transcription-quantitative polymerase chain reaction.** Gene expression of COX-2 (*PTGS2* gene) was measured using real-time quantitative polymerase chain reaction (qPCR). Specifically, human VS or GAN tissue was placed in RNA later (Qiagen, Valencia, California) and stored at  $-20^{\circ}\text{C}$  until RNA extraction. Total RNA was extracted using RNeasy Mini-Kit (Qiagen) according to the manufacturer's protocol. Quantification and quality assessment of the RNA were performed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California) or Nanodrop (ThermoScientific, Wilmington, Delaware). All samples yielded undegraded RNA as shown by electropherograms or through 260/280 nm absorbance ratios. Isolated RNA was stored at  $-80^{\circ}\text{C}$ . The RNA was reverse-transcribed to complementary DNA with TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, California) following the manufacturer's protocol. The complementary DNA was stored at either  $4^{\circ}\text{C}$  for short-term use or  $-20^{\circ}\text{C}$  for long-term storage. qPCR was performed with TaqMan primers and 6-carboxyfluorescein (6-FAM) linked fluorescent probes (Applied Biosystems) for *PTGS2* (Hs00153133\_m1) with reference gene ribosomal RNA *18s* (Hs9999901\_s1). The PCR measurements were performed using Applied Biosystems 7700 Sequence Detection System.

**Immunohistochemistry of GAN and VS specimens.** Human VS and GAN specimens were fixed in 4% paraformaldehyde for 2 hours at room temperature (RT) on shaker. The specimens were transferred to phosphate-buffered saline (PBS) and kept on shaker at  $-4^{\circ}\text{C}$  until being embedded into paraffin. Paraffin-embedded tissue on slides was deparaffinized and antigen retrieval (#S1700; Dako, Glostrup, Denmark)

was performed using manufacturer's instructions. Tissue sections on slides were placed in 5% normal horse serum with 0.4% Triton X-100 (#X-100; Sigma-Aldrich, St. Louis, Missouri) for blocking, incubated with primary antibodies against S100 (#Z031129; Dako), an SC marker, or COX-2 (#ab15191; Abcam, Cambridge, UK) at  $4^{\circ}\text{C}$  overnight, and then incubated for 2 hours at RT in secondary antibodies (Jackson-Immuno Research, West Grove, Pennsylvania). Nuclei were labeled using Hoechst 33342 stain (Invitrogen, Carlsbad, California). The sections were washed with PBS and a cover slip was mounted with VectaShield (Vector Laboratories, Burlingame, California). The tissue was visualized and imaged using a Carl Zeiss 2000 upright microscope (Carl Zeiss, Jena, Germany).

**Schwann and schwannoma cell isolation and culture.** Details of the simplified culture method are provided in Dilwali et al.<sup>17</sup> Briefly, for SC cultures, GAN samples were washed with sterile PBS thrice to remove accompanying blood or scar tissue and transferred to an equal mixture of supplemented Dulbecco's Modified Eagle's Medium (DMEM) and F12 medium, consisting of 44% DMEM (Life Technologies, Grand Island, New York), 44% F12 nutrient mixture (ThermoScientific, Waltham, Massachusetts), 10% fetal bovine serum (Life Technologies), 1% of a mixture of penicillin and streptomycin (#15140-122; ThermoScientific), and 1% GlutaMAX (Life Technologies). Nerve segments were incubated in an enzymatic mixture containing 250 U/mL hyaluronidase type I-S (Sigma-Aldrich) and 160 U/mL collagenase type I (Sigma-Aldrich) in DMEM/F12 medium for 24 hours at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  levels. No further growth factors were added. After the enzymatic incubation, the cell culture-containing medium was triturated using an 18-gauge needle (BD Biosciences, San Jose, California). Cells were recovered by centrifugation and the pellet was resuspended in supplemented DMEM/F12 medium and plated on Poly-L-lysine and Laminin precoated cover slips (BD Biosciences,). Culture medium was replaced with fresh medium after 24 hours and then every 3 days after the initial exchange.

The same protocol was followed for VS cell cultures with the only major difference being 18 hours enzymatic incubation rather than the 24 hours used here for healthy SCs.<sup>17</sup>

**Protein extraction and Western blot.** Protein levels of COX-2 were investigated semiquantitatively through Western blot analysis. Protein was extracted from VS specimens and cultures using Radioimmunoprecipitation Assay (RIPA) buffer fortified with phosphatase and protease inhibitor tablets (Roche Applied Science, Penzberg, Germany).

After quantifying the protein concentration in the tissue lysate using spectrophotometry, protein was loaded at a total protein concentration of 7.5  $\mu$ g per lane, separated on a 4%–20% Tris-glycine gel (Invitrogen), and transferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, Massachusetts). The membrane was blocked for an hour with 5% of bovine serum albumin diluted in phosphate-buffered saline with Tween 20 (PBST) (wt/vol) solution and probed with Abcam antibody against COX-2 (#ab15191; Abcam), followed by corresponding secondary antibodies (Jackson-Immuno Research). Antibody against glyceraldehyde 3-phosphate dehydrogenase (#2118; Cell Signaling, Danvers, Massachusetts) served as a total protein loading control. Membranes were visualized with an enhanced chemiluminescence detection system: ChemiDoc XRS+ (Bio-Rad Laboratories, Hercules, California). Band densities were quantified using ImageJ and were normalized to glyceraldehyde 3-phosphate dehydrogenase for a given lane.

**PTG E2 assay.** PTG E2 was assayed in the media of VS cultures and in tumor lysates using the PTG E2 Parameter Assay Kit (#KGE004B; R&D Systems, Minneapolis, Minnesota). Tumor lysates were collected by extracting total protein in PBS fortified with protease and phosphatase inhibitors. A total of 21  $\mu$ g tumor lysate protein was loaded per well. The media were collected after 48 hours of treatment from nontreated (NT) and treated cultured cells and stored at  $-80^{\circ}\text{C}$  until analysis. Manufacturer's instructions were closely followed.

**Drug preparation and treatment.** Primary VS and SC cultures were treated with aspirin (#sc-202471), NaSal (#sc-3520), and 5-ASA (#sc-202890) purchased from Santa Cruz Biotechnology (Dallas, Texas). One and 5 mM aspirin, 1, 5, and 10 mM NaSal, and 1 and 5 mM 5-ASA were prepared by mixing the appropriate amount of drug (powder form) into prewarmed culture media. The drug concentrations we used are based on the reported half maximal inhibitory concentration ( $\text{IC}_{50}$ ) values of 2.5–5 mM for aspirin-induced growth inhibition<sup>18</sup> and around 5 mM for NaSal-induced growth inhibition.<sup>19</sup> The cultures were incubated with the drugs for 48 hours. To label proliferating cells, 5-bromo-2'-deoxyuridine (BrdU) was added 20 hours before fixation. pH was measured in the media after drug addition to ensure no significant deviations. Salicylate levels in the media pretreatment were measured by high-performance liquid chromatography using a photodiode array detector at the Massachusetts General Hospital Clinical Laboratory.

**Proliferation assay.** Proliferation was assessed in cultured cells as described in Dilwali et al.<sup>17</sup> Briefly, BrdU was added to the cells at a concentration of 10  $\mu$ g/mL 20 hours before the cells were fixed. The cells were kept in the dark after the addition of BrdU. Cell and nuclear membranes were permeabilized by incubation in 1% Triton X-100 (#X-100; Sigma-Aldrich) for 10 minutes and by incubation in 2N hydrochloric acid for 20 minutes, respectively, after fixation. Primary antibodies against BrdU (#OBT0030G; AbD Serotec, Oxford, UK) and S100 (#Z031129; Dako) followed by fluorescent anti-rat and anti-rabbit immunoglobulin Gs (Life Technologies) were used. BrdU- and Hoechst-stained nuclei were counted in 3–5 fields and the ratio of BrdU-positive to Hoechst-positive nuclei was used to determine the proliferation rate in vitro. Manual counts were performed by S.D., who was blinded to treatment conditions.

**Apoptosis assay.** Apoptosis was assessed in cultured cells as described in Dilwali et al.<sup>17</sup> using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Roche Applied Science) following manufacturer's instructions. The cells were permeabilized and then incubated in TUNEL mix for 1 hour at  $37^{\circ}\text{C}$ , then for 30 minutes at RT on shaker. Nuclei were labeled with Hoechst stain. The cover slips were mounted onto slides for imaging. TUNEL- and Hoechst-stained nuclei were counted in  $\geq 3$  fields and the ratio of TUNEL-positive to Hoechst-positive nuclei was used to determine apoptosis rate in vitro. A positive control of a 10-minute DNase (Roche Applied Science) treatment before TUNEL labeling was used. Manual counts were performed by S.D., who was blinded to treatment conditions.

**MTT assays.** Cell viability was assessed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (#M-6494; Life Technologies). Cultured VS cells and GAN cells were treated with 2 mM of aspirin, NaSal, and 5-ASA, or 10  $\mu$ M COX-2 inhibitor II (EMD Millipore). Each treatment was performed in 5–6 random wells from 3 different patients. After 48 hours, 10  $\mu$ L of the 12 mM MTT was added in each well, and cells were cultured for additional 4 hours. The crystals were dissolved in 500  $\mu$ L of dimethyl sulfoxide in each well. The optical density (OD) at 540 nm of each well was detected using a photometer. The average OD value of the GAN cells exposed to vehicles (NT) was set as 100% and used to normalize OD values of the GAN cells treated with drugs. Similarly, the average OD value of the VS cells treated with vehicles (NT) was set as 100% and used

to normalize OD values of the VS cells treated with drugs. The viability in VS cells was compared with that in GAN cells and reported as percent change.

**Statistical analyses.** A 2-tailed *t* test was used to compare differences in qPCR, Western blot analyses, and PTG levels. Spearman's correlation was used to assess the relationship between PTG levels and culture growth. A paired 2-tailed *t* test was used to compare differences in proliferation and cell death after treatment with salicylates. *P* values for multiple comparisons for the different treatments were adjusted using the Benjamini-Hochberg adjustment for false discovery rate. *P* < 0.05 was considered significant for all analyses.

## RESULTS

**COX-2 is aberrantly expressed and active in VS and its derived cultures.** COX-2, an enzyme responsible for PTG synthesis, is encoded by the *PTGS2* gene. *PTGS2* was found to be 7.4-fold higher (range of 3.7–15.1, *P* = 0.02) in human VS (*n* = 9) in comparison with healthy nerves (GAN, *n* = 8), as measured through qPCR on extracted RNA from fresh human VS and GAN tissue (Fig 1, A). Furthermore, through immunohistochemistry, COX-2 was minimally detectable in 2 of 5 healthy GAN specimens: although the SCs were S100-positive as they wrapped around nerve fibers of GANs, only a few (approximately 4–5 cells per frame) COX-2-positive cells could be identified (Fig 1, B (a)). COX-2 staining was noted in most of the cytoplasmic and perinuclear regions of VS cells in 4 of 6 specimens, with 2 having a smaller COX-2 positive cell population (Fig 1, B (b)). PTGs were also detected in different tumor lysates (*n* = 5) with an average and standard deviation (SD) of  $818.9 \pm 273.4$  pg/100  $\mu$ g (Fig 1, C). Although PTGs were also detected in healthy nerves (*n* = 3) with an average and SD of  $289.4 \pm 85.2$  pg/100  $\mu$ g, the minimal values in tumors (488.8 pg/100  $\mu$ g) were higher than the maximal levels in healthy nerves (382.2 pg/100  $\mu$ g) (Fig 1, C). PTG levels in VS lysates were significantly higher than in nerve lysates (*P* = 0.019). Measuring PTGs in buffer only yielded a concentration of  $153.8 \pm 22.5$  pg/100  $\mu$ g.

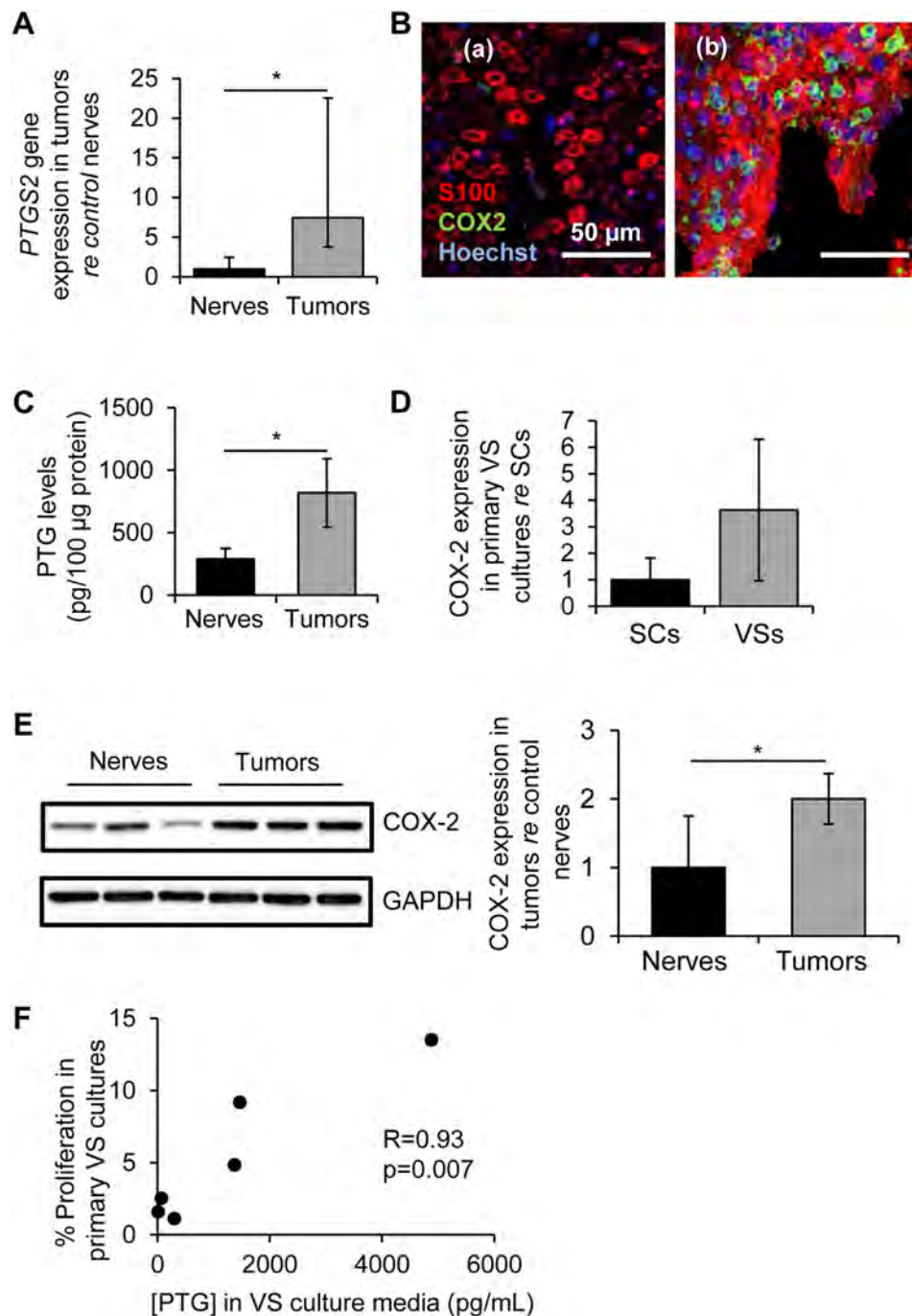
At the protein level, COX-2 was present at substantially higher levels in cultured VS cells compared with SCs. Expressed as the mean  $\pm$  SD, COX-2 expression was  $3.6 \pm 2.7$ -fold higher in cultured human VS (*n* = 6) than SCs derived from GAN ( $1.0 \pm 0.8$ , *n* = 6) as quantified through Western blot analysis (*P* = 0.06; Fig 1, D). Similar results were also observed in RIPA-extracted freshly isolated VS tumor tissues (*n* = 3) and GAN (*n* = 3) tissues. The COX-2 protein expression level in the VS tumors was  $2.05 \pm 0.82$ -

fold higher than that in the GAN tissue (*P* = 0.04; Fig 1, E). To understand the role of COX-2 in VS, we examined the correlation of PTG levels in culture media with VS cultures' growth rates, as quantified by the percentage of BrdU-positive cells in the culture. VS cultures secreted PTGs at varied levels, with an average of 1351 pg/mL and a range of 12–4880 pg/mL, and the PTG concentrations in media strongly correlated (*R* = 0.93, *P* = 0.007) with VS proliferation rate in vitro (*n* = 6; Fig 1, F).

**Salicylates reduce proliferation and viability of cultured VS cells.** To assess the therapeutic efficacy of COX-2 inhibition, we used 3 clinically relevant and well-tolerated salicylates: aspirin, NaSal, and 5-ASA. These drugs were tested on primary VS cultures established from different tumors, with *n* representing the number of different primary VS cultures used. We found that these inhibitors, used at physiologically relevant concentrations, selectively reduce VS-cultured cell proliferation. Representative images of NT cells, 5 mM aspirin-treated cells, and 1 mM NaSal-treated cells are shown in Fig 2, A (a–c), respectively. Data are summarized as average  $\pm$  standard error of the mean (SEM). Benjamini-Hochberg adjusted *P* values are provided. Proliferation is normalized to the NT cells for each culture. After 1 and 5 mM aspirin treatment, proliferation changed in VS cells to  $129.6 \pm 26.2\%$  (*n* = 4, *P* = 0.34) and  $19.3 \pm 5.5\%$  (*n* = 5, *P* = 0.00008), respectively, of the NT cells (having an SEM of 42.3%) (Fig 2, B). After 1, 5, and 10 mM NaSal treatment, VS cell proliferation changed to  $18.9 \pm 5.0\%$  (*n* = 3, *P* = 0.004),  $25.4 \pm 11.1\%$  (*n* = 7, *P* = 0.0002), and  $20.6 \pm 11.2\%$  (*n* = 6, *P* = 0.0008), respectively, of the NT cells (having an SEM of 33.4%) (Fig 2, B). After 5 and 10 mM 5-ASA treatment, VS proliferation changed to  $66.0 \pm 15.1\%$  (*n* = 6, *P* = 0.10) and  $54.8 \pm 16.5\%$  (*n* = 6, *P* = 0.03), respectively, of the NT cells (having an SEM of 36.3%) (Fig 2, B). Going from most effective to least effective based on dosage, NaSal, aspirin, and 5-ASA were all effective in reducing proliferation in VS cells.

Salicylates at these concentrations did not induce significant cell death in VS cells as measured by TUNEL staining (Fig 2, C (a–c)). After treatment with 1 and 5 mM aspirin, the cell death rate did not change, going from  $0.8 \pm 0.4\%$  in the NT cells to  $0.6 \pm 0.3\%$  (*n* = 6, *P* = 0.31) and  $2.8 \pm 2.2\%$  (*n* = 5, *P* = 0.42), respectively (Fig 2, D). Similarly, the cell death rate was not significantly affected for 5 mM NaSal and 5-ASA treated cells, going from  $1.0 \pm 0.5\%$  in the NT-cultured VS cells to  $3.3 \pm 2.3\%$  (*P* = 0.19) and  $5.6 \pm 3.6\%$  (*P* = 0.19), respectively (*n* = 5; Fig 2, D).





**Fig 1.** COX-2 is aberrantly upregulated in VS and derived primary cultures. **(A)** *PTGS2* gene expression in human VSs (n = 9 different tumors) vs GANs (n = 8 different nerves) as measured through qPCR. Error bars represent range. **(B)** Representative images of COX-2 expression (green) as visualized through immunohistochemistry in **(a)** GAN (n = 5 different nerves) and **(b)** VS (n = 6 different tumors). Schwann or schwannoma cells are labeled with S100 (red) and nuclei are labeled with Hoechst (blue). **(C)** PTG levels in tissue lysates of VS (n = 5 different tumors) and GAN (n = 3 different nerves). Error bars represent standard error of the mean. **(D)** COX-2 expression in cultured human VS (n = 6 different tumors) normalized to the expression in SC cultures (n = 6 different nerves) as quantified through Western blot analysis. Error bars represent standard deviation. **(E)** COX-2 expression in tissue specimens of VS (n = 3 different tumors) and GAN (n = 3 different nerves) assessed by Western blot analysis. Error bars represent standard deviation. **(F)** Correlation of PTG concentrations secreted in VS culture media with VS proliferation rate (% BrdU-positive cells) in vitro.  $R$  represents Spearman's correlation coefficient (n = 6 different cultures). \* $P < 0.05$ . *BrdU*, 5-bromo-2'-deoxyuridine; *COX-2*, cyclooxygenase 2; *GANs*, great auricular

Our results suggest that these salicylates are selectively cytostatic against VS cells.

Because the hypothesized mechanism of antiproliferative effect of salicylates is COX-2 inhibition, we tested a specific COX-2 inhibitor (COX-2 inhibitor II) in 3 different tumor samples. Using a substantially smaller concentration of COX-2 inhibitor II than of salicylates, we found that 10  $\mu$ M COX-2 inhibitor II reduced the proliferation in VS cells to  $48.76 \pm 11.93\%$  ( $P = 0.0007$ ), as reflected in BrdU labeling (Fig 3, A).

To further characterize the cytostatic effect of salicylates, we used the MTT assay. VS cells and GAN cells were treated with 2 mM of aspirin, NaSal, 5-ASA, or 10  $\mu$ M COX-2 inhibitor II for 48 hours. Compared with that in GAN cells, treatment with aspirin, NaSal, or 5-ASA reduced the viability of VS cells to  $70.65 \pm 6.82\%$  ( $n = 5$ ,  $P = 0.0007$ ),  $72.23 \pm 6.68\%$  ( $n = 6$ ,  $P = 0.0002$ ), or  $69.35 \pm 9.27\%$  ( $n = 5$ ,  $P = 0.002$ ), respectively, whereas COX-2 inhibitor II treatment reduced VS cell viability to  $62.58 \pm 4.95\%$  ( $n = 6$ ,  $P = 0.00001$ ) (Fig 3, B). Taken together, these data suggest that the cytostatic effect of salicylates may depend on the inhibition of COX-2.

Additionally, we measured levels of PTGs in VS to assess COX-2 inhibition. Treatment with 1 and 5 mM aspirin, and 5 mM NaSal reduced secreted PTG levels to 3.1% ( $n = 4$ ,  $P = 0.000002$ ), 3.8% ( $n = 4$ ,  $P = 0.000005$ ), and 32.2% ( $n = 3$ ,  $P = 0.07$ ) of NT cells, respectively (Fig 3, C). Our results suggest that COX-2 was inhibited after salicylate treatment.

Salicylate levels measured in culture media with 1 mM aspirin, 5 mM aspirin, 1 mM NaSal, and 5 mM NaSal, shown as the mean  $\pm$  SD were  $0.88 \pm 0.28$ ,  $3.33 \pm 1.33$ ,  $17.44 \pm 0.15$ , and  $68.24 \pm 2.61$  mg/dL, respectively. No salicylate was detected in plain media or media with 5 mM 5-ASA.

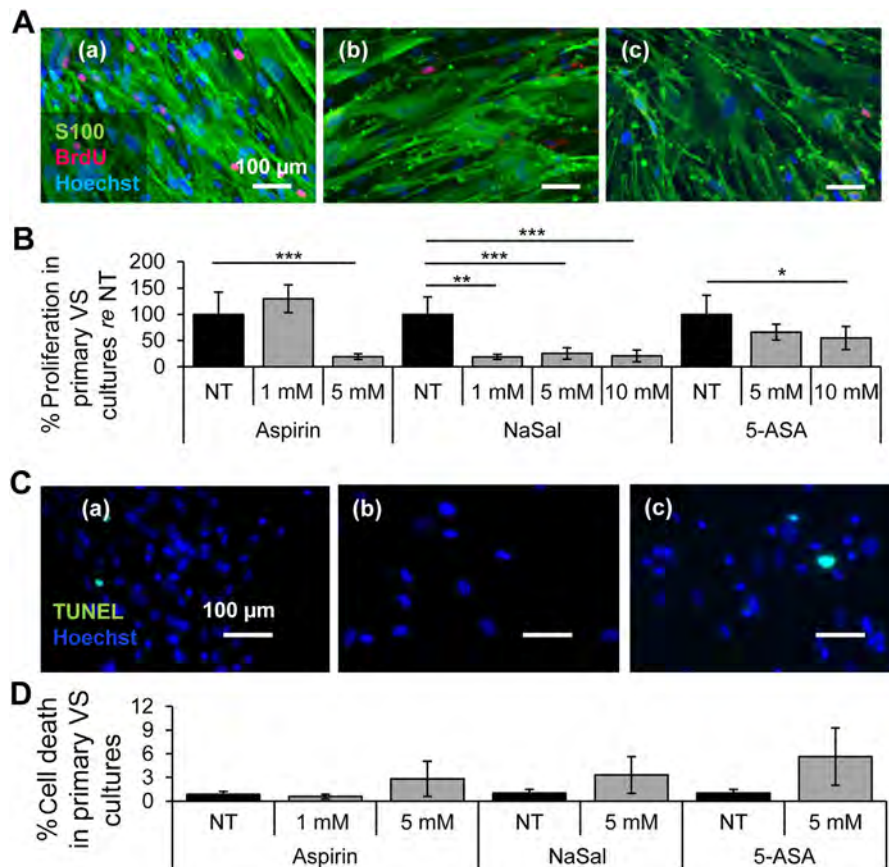
**Salicylates do not reduce proliferation of SCs.** The cytostatic effect of salicylates against VS cells seemed to be specific to the neoplastic cells because treating healthy SCs with the same concentrations of the drugs did not lead to a decrease in cell proliferation. These drugs were tested on primary SC cultures established from different GANs, with  $n$  representing the number of different primary SC cultures used. After aspirin treatment, proliferation did not change in SCs, going to  $124.4 \pm 72.9\%$  ( $P = 0.48$ ) and  $198.1 \pm 141.3\%$  ( $P = 0.47$ ) of the NT cells with 1 and 5 mM aspirin, respectively ( $n = 3$ ; Fig 3, D). After NaSal treatment, proliferation was not affected until the highest dose of

10 mM NaSal. Proliferation rate was  $104.4 \pm 13.2\%$  ( $n = 3$ ,  $P = 0.45$ ) and  $64.9 \pm 18.9\%$  ( $n = 4$ ,  $P = 0.03$ ) of the NT cells with 5 and 10 mM NaSal, respectively (Fig 3, D). After 5-ASA treatment, proliferation did not change in SCs at  $107.8 \pm 22.4\%$  ( $P = 0.51$ ) and  $109.7 \pm 26.7\%$  ( $P = 0.54$ ) of the NT cells with 5 and 10 mM 5-ASA, respectively ( $n = 3$ ; Fig 3, D). These results suggest the promising utility of salicylates to specifically target VS cells.

## DISCUSSION

We have shown that well-tolerated and clinically common salicylates led to selective decrease in proliferation and in secreted PTG levels in primary VS cultures. Our in vitro results parallel our findings of a clinical study in which we correlated the growth rates of human VSs, calculated by measuring changes in tumor size on serial magnetic resonance imaging scans, with the patient's intake of aspirin (for unrelated medical diagnoses to VS).<sup>20</sup> In that retrospective study, based on a review of the medical records over the past 32 years at our clinical center, we found that the probability of VS growth in patients who took aspirin was approximately half of that in patients with VS who did not take aspirin.<sup>20</sup> Medical records that specified aspirin dose reported oral intake of either 81 or 325 mg daily, with most (38) patients taking 81 mg for comorbidities such as cardiovascular disease. Although a low-dose aspirin (81 mg daily) may not reach the concentration in sera that we found therapeutic in our present in vitro work (1–5 mM), the acidic properties of salicylates allow them to have a high affinity toward sites of inflammation, potentially explaining their efficacy at low dosages.<sup>10</sup> Other clinical studies have shown a protective and therapeutic effect of a low dose aspirin against different types of cancers.<sup>21</sup> Although it has been known for decades that blood levels after intake of these drugs vary in humans, the therapeutic serum concentrations of the active metabolite (salicylate) that are considered adequate to treat inflammatory conditions range from 1.1 to 2.2 mM,<sup>10,22,23</sup> comparable with dosages we found efficacious in vitro. The salicylate levels measured in media with dosages that led to significant reduction in VS proliferation in vitro, being 17.4 mg/dL at 1 mM NaSal and 3.3 mg/dL at 5 mM aspirin, would be detected in serum with a dose of around 200 and 800 mg of the respective drugs.<sup>24,25</sup> This dose is less than the range of salicylate toxicity, with milder symptoms such as tinnitus being noted at

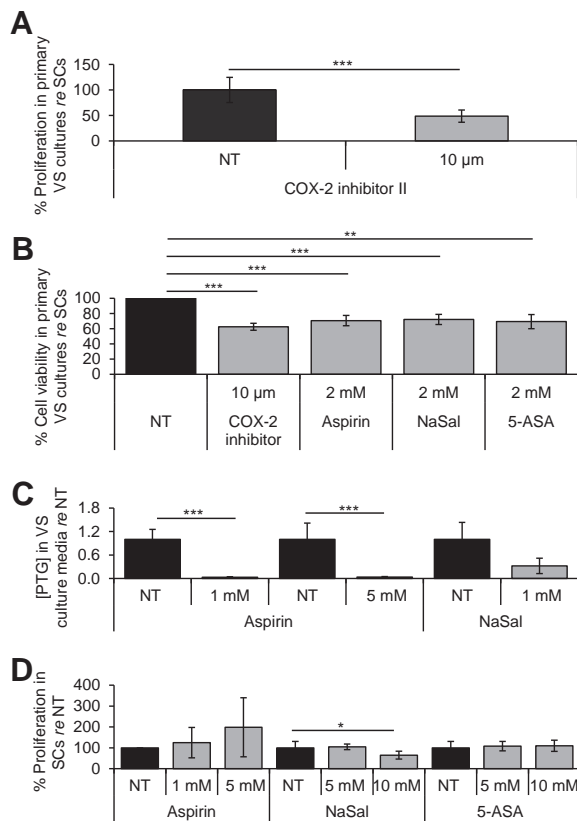
nerves; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *PTG*, prostaglandin; *qPCR*, quantitative polymerase chain reaction; *re* = in comparison with; *SCs*, Schwann cells; *VSs*, vestibular schwannomas. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.



**Fig 2.** Salicylates decrease proliferation of VS cells. (A) Representative VS culture proliferation images are shown after treatment for (a) no treatment control (NT), (b) 5 mM aspirin, and (c) 1 mM NaSal. S100 marks schwannoma cells, BrdU in nuclei marks proliferating cells. Nuclei are labeled with Hoechst. Scale bar = 100  $\mu$ m for all images. (B) Quantification of proliferation changes after treatment with aspirin, NaSal, and 5-ASA in primary VS cells normalized to proliferation in NT cells ( $n = 3-7$  different cultures). Error bars represent standard error of the mean. (C) Representative VS culture cell death images are shown after treatment for (a) NT, (b) 5 mM aspirin, and (c) 5 mM NaSal. TUNEL (green) marks dying cells. Nuclei are labeled with Hoechst. Scale bar = 100  $\mu$ m for all images. (D) Quantification of cell death rate changes after treatment with aspirin, NaSal, and 5-ASA in primary VS cells ( $n = 5-6$  different cultures for each). Error bars represent standard error of the mean. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . 5-ASA, 5-aminosalicylic acid; BrdU, 5-bromo-2'-deoxyuridine; NaSal, sodium salicylate; re = in comparison with; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VSs, vestibular schwannomas. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

approximately 25–35 mg/dL serum salicylate levels.<sup>26</sup> Because of the simplified nature of a culture model, it is difficult to directly translate the concentration effective on cultured tumor cells with the concentration required in vivo to be efficacious when administered systemically. To gain some insight into whether these concentrations would be feasible in vivo, we applied salicylates onto healthy SCs. We did not find a decrease in SC proliferation with salicylates, suggesting the dosages to be tolerable to SCs. Additionally, salicylates readily cross the blood-brain barrier and can reach up to 50% of the concentration present in the blood,<sup>27</sup> an appealing aspect that makes translation of salicylates against VS even more promising. Regardless, salicylate

concentrations in tumor tissue are likely to be similar to those in serum because the blood-brain barrier is compromised in intracranial tumors.<sup>28</sup> Nonetheless, because NSAID concentrations effective against VSs in vivo have not been established, it would be important to define drug dosage curves for NSAIDs in vivo through the use of mouse models or phase 0 trials in humans. Further, the use of a specific COX-2 inhibitor, COX-2 inhibitor II, at a concentration of 10  $\mu$ M also led to decreased proliferation of cultured VS cells, suggesting that clinically relevant specific COX-2 inhibitors, such as celecoxib, could be effective at even lower dosages and may be more so well tolerated than NSAIDs at the  $\geq 1$  mM dosages efficacious in our VS culture work.



**Fig 3.** Salicylates and a specific COX-2 inhibitor II decrease VS cell viability but do not affect proliferation of normal SCs. (A) Quantification of proliferation changes after treatment with COX-2 inhibitor II ( $n = 5$  different cultures). Error bars represent standard deviation. (B) MTT assays of cell viability after treatment of GAN cells and VS cells with 2 mM aspirin, 2 mM NaSal, 2 mM 5-ASA, and 10 μM COX-2 inhibitor II (5–6 different wells from 3 different samples for each treatment). Error bar represents SD. There is no error bar associated with NT because it was set to 100% for every comparison of NT VS and GAN cells. (C) Secreted PTG levels in VS culture media after treatment for NT, 1 and 5 mM aspirin, and 5 mM NaSal ( $n = 3$ –4 different cultures). Error bars represent standard deviation. (D) Quantification of proliferation changes after treatment with aspirin, NaSal, and 5-ASA in primary SCs normalized to proliferation in NT cells ( $n = 3$ –4 different cultures). Error bars represent standard error of the mean. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . 5-ASA, 5-aminosalicylic acid; *BrdU*, 5-bromo-2'-deoxyuridine; COX-2, cyclooxygenase 2; GANs, great auricular nerves; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaSal, sodium salicylate; NT, nontreated; PTG, prostaglandin; re = in comparison with; SCs, Schwann cells; VSs, vestibular schwannomas.

The significant correlation of PTG levels with VS culture proliferation rate is in line with the previous literature that COX-2 expression correlated with VS growth rate.<sup>8</sup> Further, substantially decreased PTG levels in the media after salicylate treatment suggest that the salicylates led to COX-2 inhibition. It is interesting that the salicylate effect was cytostatic but not cytotoxic in VS cells. Although salicylates can be both cytostatic

and cytotoxic in neoplastic cells, most studies implicate salicylate-mediated cytotoxic effect to mechanisms other than COX-2 inhibition.<sup>29</sup> In our case, salicylates may have a different therapeutic window for cytotoxic than for cytostatic effects in VS cells; we did not test higher salicylate concentrations because they would be greater than the range considered safe in vivo.

Interestingly, salicylate was not detected in media with 5-ASA, suggesting that 5-ASA may be acting through an alternative active metabolite to inhibit VS proliferation. Further, as we have only shown a correlative decrease in PTG levels with salicylate application, it is feasible that the salicylates could be acting through other molecular pathways along with COX-2 inhibition to lead to VS cytostaticity as salicylates do have multiple targets. For instance, although COX-2 is a preferential target for salicylates compared with COX-1,<sup>9</sup> it is possible that COX-1 is also inhibited in VS cells as COX-1 expression and activity was not assessed in this study. Additionally, aspirin and NaSal can also inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) directly, through blockade of I kappa B kinase (IκK), especially at higher dosages ( $\geq 5$  mM).<sup>30–32</sup> Aspirin may operate through this mechanism in our work, as we do not note decreased proliferation at 1 mM aspirin, although PTG secretion is inhibited significantly. Interestingly, the COX-2 gene promoter does have a κB binding site<sup>9</sup> and it could be that NSAID inhibiting NF-κB-driven cell proliferation is ultimately because of a decrease in COX-2 expression.

It has also been shown that celecoxib, a COX-2-specific inhibitor, could induce apoptosis in colon cancer lines by inhibiting the 3-phosphoinositide-dependent kinase 1 (PDK-1) activity.<sup>33</sup> PDK-1 is an upstream molecule of AKT; it can phosphorylate AKT and induce AKT activities.<sup>34</sup> PDK-1 is also involved in NF-κB activation.<sup>35</sup> These results in colon cancer cells indicated that PDK-1 and AKT are both involved in the proliferation of tumor cells. By analogy, similar pathways may be regulating the growth of VS. Indeed, it has been shown that the promotion of VS invasion by epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) was modulated by AKT.<sup>36</sup> OSU-03012, a PDK-1 inhibitor developed at Ohio State University, had growth inhibitory and antitumor activities on VS and malignant schwannoma cells,<sup>37</sup> suggesting that PDK-1 can promote VS growth.

Although COX-2 inhibition does not seem to lead to significant adverse effects, COX-1 inhibition can interfere with homeostatic functions, which may cause increasing incidence of gastrointestinal hemorrhage and ulceration with chronic intake.<sup>9</sup> Among the salicylates tested, aspirin is a more potent drug. It leads to an



irreversible inhibition of COX enzymes by acetylating their binding sites, whereas NaSal and 5-ASA inhibit COX enzymes through reversible competitive binding.<sup>9,10</sup> We tested NaSal and 5-ASA because they can serve as alternatives to aspirin for people with hypersensitivity to aspirin. Our results also motivate trials of COX-2-selective inhibitors such as celecoxib against VS as these compounds further curb the adverse effects of general COX inhibitors.<sup>9</sup>

Our preclinical data motivate future work studying the mechanisms behind the therapeutic efficacy of salicylates against VS cells and clinical translation of these drugs against VS. We have established the aberrance of COX-2 in VS and VS cultures. The secreted levels of its enzymatic product, PTGs, correlated with VS culture proliferation rates. We found clinically well-tolerated COX-2 inhibitors, namely aspirin, NaSal, and 5-ASA, to minimize proliferation of VS cells, without affecting healthy SCs. Our in vitro findings corroborate our retrospective clinical observation that the probability of VS growth decreased to approximately half in patients taking aspirin.<sup>20</sup> To the best of our knowledge, salicylates would be the most promising treatments against sporadic VS as they are commonly used for a variety of pathologies, including other tumors such as colon cancer, with minimal adverse effects when used within the clinically well-established therapeutic range. For the histologically nonmalignant VSs, the cytostatic effect alone, without the cytotoxic effect, would be therapeutic.

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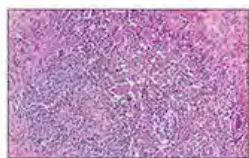
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# Interplay between VEGF-A and cMET signaling in human vestibular schwannomas and schwann cells

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**Keywords:** hepatocyte growth factor, Schwann cells, siRNA, cross-talk, vascular endothelial growth factor, vestibular schwannoma

**Abbreviations:** BrdU, 5-Bromo-2'-Deoxyuridine; cMET, MNNG HOS transforming gene, hepatocyte growth factor receptor; DMSO, Dimethyl sulfoxide; GAN, Great auricular nerve; HCl, Hydrochloric acid; HGF, Hepatocyte growth factor; HGF, Gene encoding HGF protein; HRP, Horse-radish peroxidase; KDR, Gene encoding vascular endothelial growth factor receptor 2; MET, Gene encoding cMET protein; mRNA, Messenger ribonucleic acid; NF2, Neurofibromatosis type 2; PBS, Phosphate buffered saline; S100, Schwann cell/schwannoma cell marker; SD, Standard deviation; SEM, Standard error of mean; siRNA, Small interfering ribonucleic acid; VEGFA, Gene encoding VEGF-A protein; VEGF-A, Vascular endothelial growth factor-A; VEGFR2, Vascular endothelial growth factor receptor 2; VS, Vestibular schwannoma.

Vestibular schwannoma (VS), the fourth most common intracranial tumor, arises from the Schwann cells of the vestibular nerve. Although several pathways have been independently implicated in VS pathobiology, interactions among these pathways have not been explored in depth. We have investigated the potential cross-talk between hepatocyte growth factor (HGF) and vascular endothelial growth factor-A (VEGF-A) in human VS, an interaction that has been described in other physiological and pathological cell types. We affirmed previous findings that VEGF-A signaling is aberrantly upregulated in VS, and established that expression of HGF and its receptor cMET is also significantly higher in sporadic VS than in healthy nerves. In primary human VS and Schwann cell cultures, we found that VEGF-A and HGF signaling pathways modulate each other. siRNAs targeting cMET decreased both cMET and VEGF-A protein levels, and siRNAs targeting VEGF-A reduced cMET expression. Additionally, siRNA-mediated knockdown of VEGF-A or cMET and pharmacologic inhibition of cMET decreased cellular proliferation in primary human VS cultures. Our data suggest cross-talk between these 2 prominent pathways in VS and highlight the HGF/cMET pathway as an additional important therapeutic target in VS.

## Introduction

Vestibular schwannomas (VSs), benign tumors arising from Schwann cells of vestibular nerves, are the fourth most common intracranial tumors.<sup>1</sup> Although several pathways have been implicated in VS pathobiology, interactions among these pathways have been scarcely established. Levels of vascular endothelial growth factor-A (VEGF-A), a prominent mitogenic and angiogenic factor, and its receptor tyrosine kinase VEGFR1 correlate with VS growth rate.<sup>2</sup> Administration of bevacizumab, a humanized VEGF-A antibody, to patients with Neurofibromatosis type 2 (NF2)-associated VS led to a volumetric decrease in 55% of the VSs.<sup>3,4</sup> As investigators continue to elucidate all of the factors that interact with VEGF-A, it is important to explore the potential of VEGF-A to regulate and be regulated by

other molecules that could be driving VS growth, providing us with new therapeutic targets and the ability to overcome potential drug resistance inevitable with monotherapies.

HGF, a potent angiogenic factor, and its receptor tyrosine kinase cMET have been implicated in several other cancers<sup>5</sup> in addition to VS,<sup>6</sup> though they have previously not been explored as therapeutic targets against VS. We investigated cross-talk between VEGF-A and HGF, an interaction that has been established in a few other cell types. The HGF/cMET signaling pathway has been shown to interact closely with the VEGF-A signaling pathway in other physiological signaling, such as in endothelial cells,<sup>7</sup> and in pathological signaling, such as in adenocarcinoma<sup>8</sup> and glioma cells.<sup>9</sup> Specifically, previous research in endothelial cells shows that VEGF-A and HGF synergistically activate mitogen-activated protein kinases (MAPKs), stimulation

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with VEGF-A increases cMET levels, and stimulation with HGF elevates VEGFR2 levels.<sup>7</sup> Exploring this cross-talk between VEGF-A and cMET in VS cells, compared to non-neoplastic Schwann cell controls, can provide insight into the mechanism for VEGF-A and HGF signaling-mediated VS growth. Further, with a focus on devising therapies aimed toward reducing functional HGF or VEGF-A signaling, we silenced cMET or VEGF-A and noted the resultant effect on the other factor. These experiments contrast previous work focused on establishing cross-talk through increased signaling of these pathways.

We found that HGF signaling, along with VEGF-A signaling, is significantly upregulated in VS, as measured through mRNA and secreted protein levels. In both primary human VS and SC cultures, we found that VEGF-A and cMET signaling pathways modulate each other. In VS cultures, siRNAs targeting cMET decreased VEGF-A and VEGFR2 protein levels, and targeting VEGF-A reduced cMET expression. Additionally, siRNA-mediated knockdown of VEGF-A or cMET, and pharmacologic inhibition of cMET led to decreased proliferation in primary VS cultures. In this study, by investigating the cross-talk between VEGF-A and cMET pathways in VS, we highlight cMET as an additional therapeutic target.

## Materials and Methods

### Specimen collection

Freshly-harvested human specimens of sporadic VSs and GANs were collected from indicated surgeries. The study protocols were approved by Human Studies Committee of Massachusetts General Hospital (Protocol No. 2004- P2297/2, PI: K.M.S) and the Massachusetts Eye and Ear Infirmary (Protocol No. 05-02-009X, PtdIns: K.M.S). Written informed consent was received from all subjects prior to inclusion in the study. Immediately after extraction, specimens were placed in saline solution and transported to the laboratory on ice. The specimen was rinsed with saline and divided for protein, RNA or culture work. Procedures were in accordance with the Helsinki Declaration of 1975.

### Real time-quantitative polymerase chain reaction (RT-qPCR)

Expression of VEGF and HGF pathway was measured in VSs versus GANs (healthy nerve controls). Specifically, human VS or GAN tissue was placed in RNAlater (Qiagen, #76106) and stored at  $-20^{\circ}\text{C}$  until RNA extraction. Total RNA was extracted using RNeasy Mini Kit (Qiagen, #74104) according to the manufacturer's protocol. Quantification and quality assessment of the RNA were performed using Agilent 2100 Bioanalyzer (Agilent Technologies) or NanoDrop (ThermoScientific). All samples yielded undegraded RNA as shown by electropherograms or through 260/280 nm absorbance ratios. Isolated RNA was stored at  $-80^{\circ}\text{C}$ . The RNA was reverse-transcribed to cDNA with TaqMan Reverse Transcription Reagent Kit (Applied Biosystems, #4304134) following the manufacturer's protocol. The cDNA was stored at either  $4^{\circ}\text{C}$  for short term or  $-20^{\circ}\text{C}$  for long term.

qPCR was performed with TaqMan primers and 6-Carboxy-fluorescein (6-FAM) linked fluorescent probes (Applied Biosystems) for VEGFA (#Hs00900055\_m1), HGF (#Hs00300159\_m1), KDR (#Hs00911700\_m1) and MET (#Hs01565582\_g1). The reference gene was rRNA 18s (#Hs9999901\_s1). Statistical significance was determined using the 2-tailed t-test with a  $P < 0.05$  considered significant after a Benjamini-Hochberg correction for multiple hypotheses.

### Protein extraction and immunoblotting

Translation and activation of the VEGF and HGF pathway components was investigated through western blot analysis. Total protein was extracted from freshly-harvested specimens of VS and GAN in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors on ice. The lysate was isolated by centrifugation at 10,000 RPM for 10 minutes at  $4^{\circ}\text{C}$ . The protein was stored at  $-80^{\circ}\text{C}$  and was subjected to a maximum of 2 freeze/thaw cycles. Samples were loaded at a total protein concentration of 7.5–15  $\mu\text{g}$  per lane, separated on a 4–20% Tris-Glycine Gel (Life Technologies, #EC6025BOX) and transferred onto Immobilon-P PVDF Membrane (Millipore, #IPVH00010). The membrane was blocked for an hour with 5% Bovine Serum Albumin/PBST (w/v) solution and probed with Santa Cruz antibodies against VEGF (#sc-152) and cMET (#sc-161) and Cell Signaling antibodies against phosphorylated (P)-cMET (#3077) and VEGFR2 (#2479). Antibody against  $\beta$ -actin (Cell Signaling, #4970) served as an internal control. Membranes were visualized with an enhanced chemiluminescence detection system ChemiDoc Plus (BioRad Laboratories). Band densities were quantified using ImageJ and were normalized to  $\beta$ -actin for a given lane. Statistical significance of the data was determined using the 2-tailed t-test with a  $p < 0.05$  considered significant.

### Cytokine array

Detailed methods have been published previously.<sup>14</sup> Briefly, VS and GAN secretions were collected by incubating freshly resected and washed tissue in PBS for 1 hour at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  levels. Human cytokine array membranes (RayBiotech, Inc., custom order) were probed with 21 VS secretion samples, 7 GAN samples and 1 blank sterile PBS. Manufacturer's protocol was followed in conducting the experiment and data analysis. Samples were dialyzed twice with PBS. The membranes were exposed to the blocking buffer at room temperature for 1 hour, incubated with sample at  $4^{\circ}\text{C}$  overnight, washed with wash buffer I and II at room temperature, incubated with biotin-conjugated antibodies at  $4^{\circ}\text{C}$  overnight, washed and incubated with HRP-conjugated streptavidin at room temperature for 1 h. The membranes were then exposed in ChemiDoc (BioRad Laboratories). The relative expression levels of HGF and VEGF were compared after densitometry analysis using Quantity One (BioRad Laboratories). Statistical significance was determined using ANOVA test with  $\alpha$  set to 0.05.



## Primary human Schwann cell and vestibular schwannoma cell culture

Detailed methods have been published previously.<sup>15</sup> Briefly, using sterile technique under the hood, freshly harvested VS or GAN tissue was rinsed in sterile PBS twice and cut into 1 mm-sized pieces in Dulbecco's modified eagle's medium with Ham's F12 nutrient mixture (DMEM/F12), 10% fetal bovine serum, 1% Penicillin/Streptomycin (Pen/Strep) and 1% GlutaMAX (all purchased from Life Technologies). To obtain a more pure SC population, the epineurium was removed from the nerve tissue by tugging and removing the outer layers under a dissecting microscope. The specimen pieces in the media were centrifuged at 3000 g at 8°C for 3 minutes. The media was aspirated and the tissue pellet was incubated in new media containing 5% Collagenase (Sigma-Aldrich, #C1639) and 0.5% Hyaluronidase (Sigma-Aldrich, #H3506) for 18–24 hours at 37°C. The cells were plated in Poly-L-Ornithine and Laminin pre-coated 12-well culture dishes with 5 mm glass slides (BD Biosciences, #354087) in DMEM/F12 media with 10% FBS, 1% Pen/Strep and 1% glutamine. The cell cultures were maintained for 3 to 4 weeks and media was changed every 3 d

## siRNA and pharmacologic treatment

To understand cross-talk between HGF and VEGF-A pathway, cultured VS cells were incubated with Ambion siRNAs targeting VEGF (#s461), MET (#s8700) or KDR (#s7824). To understand whether HGF signaling contributed to VS proliferation, cultured VS cells were treated with MET inhibitor SU11274 (Sigma-Aldrich, #S9820). Seventy-two hours after siRNA treatment or 12 hours after treatment with 2  $\mu$ M SU11274, cells were incubated with 10  $\mu$ g/mL 5-Bromo-2'-Deoxyuridine (BrdU, Life Technologies, #B23151) for 20 hours. After treatment, cells were fixed with 4% paraformaldehyde. After cell membrane permeabilization by incubation in 1% Triton-X, cells were incubated in 1N HCl for 25 mins, blocked for 1 hour in normal horse serum (NHS, Sigma-Aldrich) and incubated with primary antibodies against BrdU (AbD Serotec, #OBT0030G) and S100 (Dako, #Z0311) diluted in NHS overnight at 4°C. After PBS washes, secondary antibodies (Alexa Fluor 555 anti-rat and Alexa Fluor 488 anti-rabbit, Life Technologies) were applied for 1 hour at room temperature and the cells' nuclei were counterstained with Hoechst stain (Life Technologies, #H1399). The coverslips were mounted onto glass slides for fluorescence microscopy. Cells were counted by an investigator (D.S.R or SD) blinded to the treatment conditions. Nuclei were counted in  $\geq 3$  fields and the cell proliferation rate was calculated as BrdU positive nuclei over the total number of nuclei. The inhibitors' effect on proliferation was normalized to the corresponding tumor's proliferation in culture receiving no treatment (DMSO only). A paired 2-tailed t-test was performed to compare the control and treatment groups with  $P < 0.05$  considered statistically significant.

## Results

### Increased expression and activation of cMET and VEGF-A signaling in VS

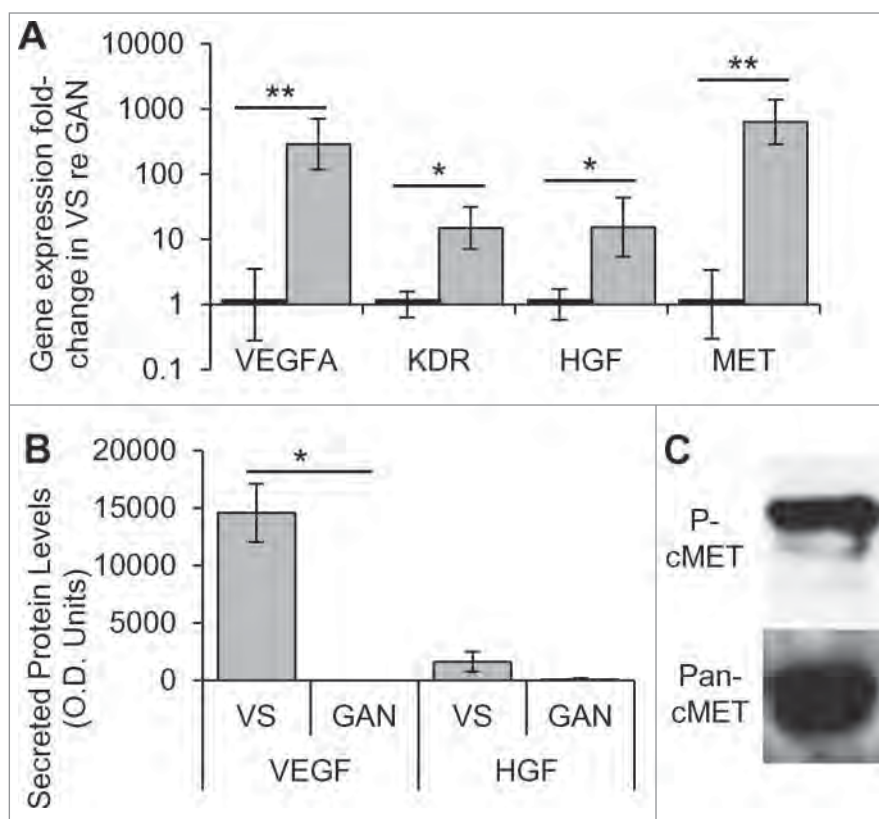
To investigate aberrant expression of HGF and VEGF-A signaling pathways in VSs, gene expression differences in HGF, VEGF-A, cMET, and VEGFR2 (gene KDR) were determined in human VSs in comparison to healthy nerves. Tumor specimens ( $n = 8$  different specimens) had significantly elevated expression of the HGF and VEGF signaling pathways compared to healthy nerves ( $n = 7$  different specimens), being 286.7-fold ( $p = 0.003$ ) and 15.0-fold ( $p = 0.011$ ) higher with a standard error range of 116.6–705.4 and 7.1–31.6 for VEGFA and KDR, respectively (Fig. 1A). Healthy nerves had a range of 0.3–3.5 and 0.6–1.6 for VEGFA and KDR, respectively. HGF and its receptor MET were also significantly upregulated, being 15.4-fold ( $p = 0.043$ ) and 632.0-fold ( $p = 0.001$ ) with a range of 5.4–43.7 and 286.0–1391.9, respectively. Healthy nerves had a standard error range of 0.6–1.7 and 0.3–3.4 for HGF and MET, respectively (Fig. 1A).

We sought to determine if VEGF-A or HGF secretion levels were higher in VSs than in healthy nerves. Measuring HGF and VEGF-A levels using a cytokine array, we found VEGF-A to be selectively secreted from VSs ( $n = 21$  different tumors), with an average optical density (O.D.) value of  $14,558 \pm$  standard error of mean (SEM) of 2,527, and no detectable VEGF-A in great auricular nerve (GAN) secretions ( $n = 7$  different nerves) (Fig. 1B). This differential level of secretion was highly significant ( $p = 0.003$ ). HGF tended to be secreted at higher levels in VSs, with an O.D. value of  $1,615 \pm 885$ , than in GANs, which had an O.D. value of  $87 \pm 87$ , although the trend did not meet significance ( $p = 0.334$ , Fig. 1B).

To understand if cMET is activated in sporadic VSs, we investigated the phosphorylation status of c-MET at the Tyrosine 1234 site. Five independent sporadic VS tumors consistently demonstrated phosphorylation of cMET (Fig. 1C).

### Cross-talk between cMET and VEGF-A signaling pathways in primary SCs

Previous studies in endothelial cells identified possible cell signaling cross-talk between the VEGF-A and HGF receptor signaling pathways.<sup>7,9</sup> We have investigated this cross-talk in normal SCs with siRNAs targeting the VEGF-A and HGF signaling pathways. Comparing protein expression in SCs from the same culture treated with vehicle only, siRNAs were capable of knocking down VEGF-A and cMET substantially, to  $32 \pm 25\%$  ( $n = 4$  different cultures,  $p = 0.04$ ) and  $54 \pm 22\%$  ( $n = 5$  different cultures,  $p = 0.003$ ) of vehicle-only treated cells, respectively (Fig. 2A and B). Importantly, MET knockdown decreased VEGF-A levels in normal SCs significantly, reducing the protein levels to  $43 \pm 34\%$  of vehicle only-treated cells ( $p = 0.02$ ,  $n = 4$  different cultures, Fig. 2A and B). MET knockdown also decreased VEGFR2 levels, reducing the protein levels to  $50 \pm 24\%$  ( $n = 3$  different cultures, Fig. 2A and B) of vehicle only-treated cells, although this trend did not meet significance ( $p = 0.07$ ).



**Figure 1.** HGF and VEGF-A pathways are aberrantly expressed and activated in VS. **(A)** Gene expression of VEGFA and its receptor KDR, and HGF and its receptor MET, in human VS (n = 8 different tumors) normalized to great auricular nerves (GAN, n = 7 different nerves) as measured through qPCR. \* $P < 0.05$ , \*\* $P < 0.01$ . Error bars represent range. **(B)** VEGF and HGF protein levels in secretions from human VS (n = 21) and GAN (n = 8). \* $P < 0.05$ . **(C)** Representative image of cMET expression and phosphorylation (Try 1234, P-cMet) in VS, as detected by protein gel blot (n = 5). re = in comparison to.

VEGFA knockdown did not lead to a significant decrease in cMET levels, at  $28 \pm 32\%$  of vehicle only controls (n = 2 different cultures,  $p = 0.19$ ), or of VEGFR2 levels, at  $70 \pm 43\%$  with VEGFA siRNA treatment of vehicle only controls (n = 3 different cultures,  $p = 0.35$ ) (Fig. 2A and B). Although VEGFR2 level changes were not consistent between experiments with VEGFA siRNA leading to a large variability, c-MET levels were consistently decreased in the 2 experiments. More experiments with VEGFA siRNA in SCs will be helpful in establishing the protein expression changes with confidence.

#### Cross-talk between cMET and VEGF-A signaling pathways in primary VS cells

Knockdown of MET also led to decreased VEGF-A and VEGFR2 in primary VS cultures as noted in SCs. Comparing protein expression in VS cells from the same culture treated with vehicle only, siRNAs were capable of knocking down VEGFA and MET to a significant extent, with  $48 \pm 25\%$  (n = 5 different cultures,  $p = 0.009$ ) and  $51 \pm 30\%$  (n = 6,  $p = 0.005$ ) knockdown achieved, respectively (Fig. 2B). MET knockdown led to a decrease in VEGF-A and VEGFR2 levels, with reduction to

$62 \pm 31\%$  ( $p = 0.03$ ) and  $48 \pm 16\%$  respectively (n = 5 different cultures,  $p = 0.007$ , Fig. 2B). VEGFA knockdown did cause a significant decrease in cMET expression in VS cells, reducing cMET levels to  $62 \pm 29\%$  of the controls ( $p = 0.04$ , Fig. 2B).

#### Decreased VS cell proliferation with molecular VEGFA and MET or pharmacologic cMET inhibition

To understand the implications of silencing VEGFA and MET in VS cells, cell proliferation studies were performed in primary VS cells. Basal cell proliferation for VS cells treated with vehicle only was 14% on average, with a range of 2.2 to 7.0% (Fig. 2C (a)). Silencing VEGFA or MET reduced VS cell proliferation to  $29.7 \pm 1.8\%$  (SEM) (n = 5 different cultures,  $p < 0.01$ , Fig. 2C (b), 1D) and  $34.8 \pm 11.4\%$  (n = 5 different cultures,  $p = 0.02$ , Fig. 2C (c), 2D) of control. Similarly, specific inhibition of cMET signaling with the 2  $\mu$ M cMET inhibitor SU11274 reduced proliferation to  $22.4 \pm 11.7\%$  (Fig. 2C (e), 2D) of VS cells treated with DMSO only ( $9.1 \pm 3.4\%$ ) (n = 4 different cultures,  $p < 0.01$ , Fig. 2C (d)).

#### Discussion

To gain a deeper understanding of the VS pathobiological interactome, we have focused on investigating the relationship between the HGF and VEGF-A signaling pathways. Our work establishes abnormal upregulation and activation of the HGF pathway in VS pathobiology. We found significantly higher levels of HGF and cMET being transcribed in comparison to healthy nerves, activated phosphorylated cMET in all tumors tested, and relatively higher levels of secreted HGF. Our findings further expand on previous work showing that HGF and cMET are present in VSs based on immunohistochemical staining of human VS surgical specimens.<sup>6</sup> Importantly, we found that siRNA-mediated MET silencing or pharmacological inhibition of cMET led to significantly decreased primary VS cell proliferation, demonstrating cMET as a novel therapeutic target. Targeting of cMET and HGF may provide an alternate or adjuvant therapy to other pharmacological approaches being investigated to modulate VS growth.

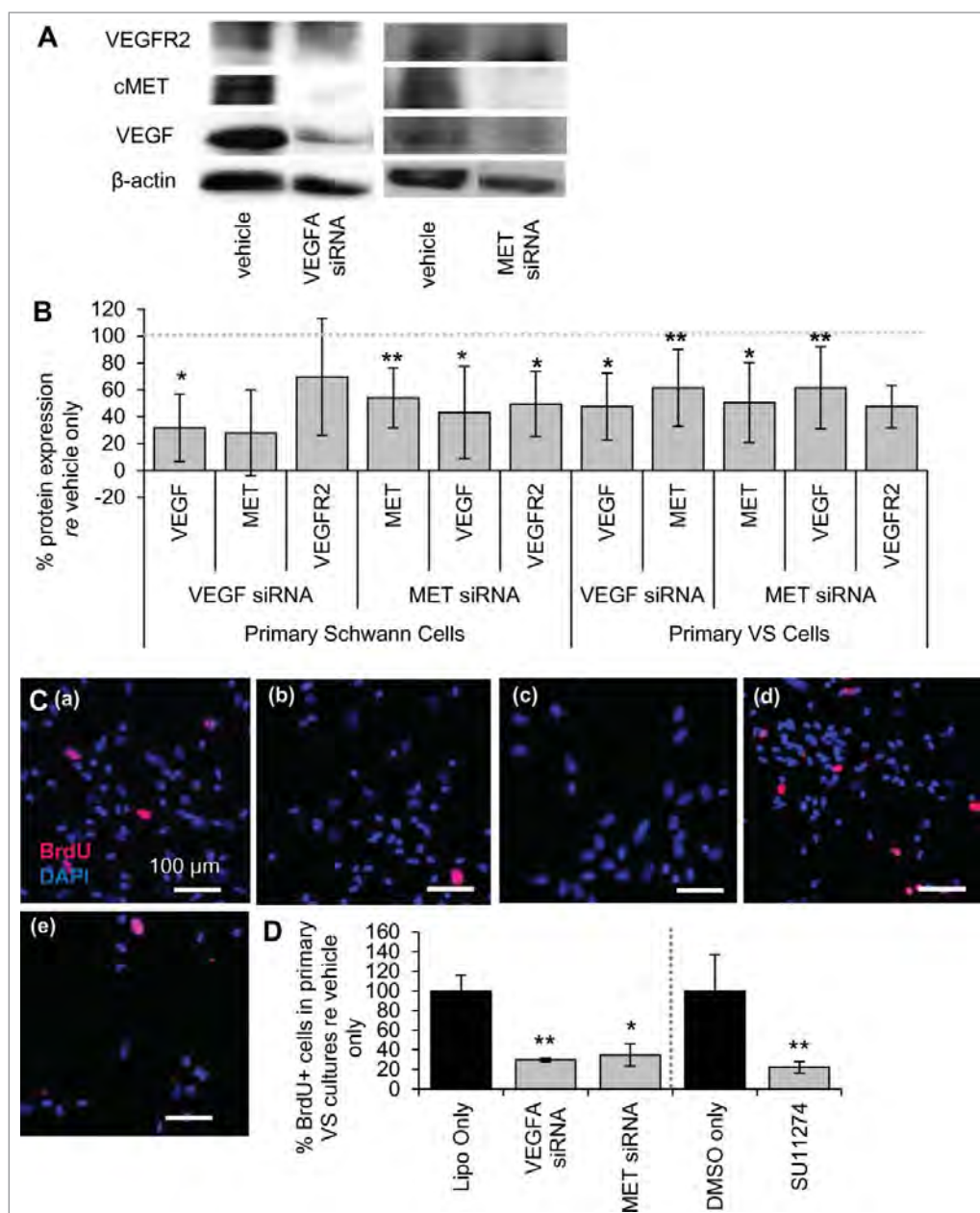
In this work, we also affirmed aberrant VEGF-A signaling as VEGF-A and VEGFR2 were expressed at significantly higher levels in VS and VEGF-A was secreted at significant higher, albeit variable, levels in VS in comparison to healthy nerves. We also confirmed VEGF-A's role in VS growth as siRNA-mediated VEGFA silencing inhibited primary VS cell proliferation. This

observation is in agreement with previous work showing the therapeutic efficacy of bevacizumab in VS patients with neurofibromatosis type 2 (NF2) and in mice cranially xenografted with the NF2 cell line HEI-193.<sup>10</sup> We also show efficacy of anti-VEGF-A therapy against sporadic VS cells.

Along with exploring VEGF-A and cMET's role in VS separately, we explored cross-talk between these 2 pathways. Previous exploration of the cross-talk in other cell types<sup>7,9</sup> focused on changes due to the elevation of either HGF or VEGF-A in cultures. With a focus on devising therapies and therefore reducing functional HGF or VEGF-A signaling, we designed our study to silence cMET or VEGF-A and note the effect on the other factor. VEGF-A and cMET have a direct regulatory relationship, rather than inverse, in VS and SCs. The trends discovered in VS and SC cultures are in line with previous studies in other cell types. Further, since decreases in VEGFR2 after VEGF siRNA treatment were not observed in SCs, it suggests that the siRNA targets its intended target specifically.

The stage of VEGF and cMET cross-regulation seems to be at the transcriptional level, in which VEGF-A and HGF signaling regulates downstream gene expression ultimately leading to modulation of the other pathway. Sul-pice et al. demonstrated that, both VEGF-A and HGF slightly increased the MET and KDR mRNA levels, respectively, in endothelial cells.<sup>7</sup> Moriyama et al. also found a similar pattern in which treatment of the glioma cells with HGF lead to increased secretion of VEGF proteins accompanying increased transcription of VEGF mRNA in a dose-dependent fashion.<sup>9</sup> This

relationship could also be potentially investigated in SC and VS cultures by measuring VEGFR2 and MET mRNA levels after HGF and VEGFA siRNA treatment.



**Figure 2.** VEGF-A and cMET pathways interact at the molecular level. (A) Representative image of western blot showing expression of VEGFR2, cMET and VEGF-A for vehicle only and for siRNAs targeting VEGFA and MET genes in primary human SCs. (B) Protein expression of VEGF-A, cMET and VEGFR2 after VEGFA and MET siRNA treatment of cultured human SCs (n = 2–4 different cultures) and VS cells (n = 5 different cultures) quantified through protein gel blot analysis. All levels are normalized to vehicle only protein expression, being 100% (dashed line). \* $P < 0.05$  \*\* $P < 0.01$ . Error bars represent SEM. (C) Representative pictures of primary human VS cells treated with (a) vehicle only, (b) VEGFA siRNA, (c) MET siRNA, (d) DMSO only or (e) SU11274. BrdU in nuclei (red) marks proliferating cells, nuclei are labeled with DAPI. Scale bar = 100  $\mu$ m for all images. (D) Quantification of proliferation changes after siRNA (n = 5 different cultures) and after SU11274 (n = 4 different cultures) treatment of primary VS cells normalized to proliferation in control non-treated cells. \* $P < 0.05$ , \*\* $P < 0.01$ . re = in comparison to. Error bars represent SEM.



We also found that MET siRNA led to a decrease in VEGFR2 levels in both primary SCs and VS cells, a correlation that has not been published for any cell type thus far. This result is intriguing because through its regulation of protein kinase B, p38 and other kinases downstream, the HGF/cMET signaling pathway may modulate several transcription factors that could in turn regulate many genes including VEGFA and KDR.<sup>7,9</sup> Our findings could explain, at least partially, how neoplastic cells sustain growth and survival in an autocrine manner.

The cross-talk between VEGF and HGF pathways in VS is fascinating as it suggests that decreasing VEGF-A levels through pharmacological means, such as bevacizumab, could modulate cMET levels. VEGF-A has been known to cross-talk with several other biological molecules, such as fibroblast growth factor 2.<sup>11</sup> VEGF-A inhibition may also affect several of these pathways in VS cells, with the cumulative effect of relatively high therapeutic efficacy of anti-VEGF therapy against VS, as seen with the clinical use of bevacizumab. To understand bevacizumab's effect on the entire VS pathobiological interactome, future studies are needed to assess proteomic and transcriptomic changes in VS after bevacizumab treatment.

Finally, our work provides insight into potential drug resistance in VSs. Resistance against bevacizumab has been noted in other tumors and cancers, but has yet to be explored after long-term use in VS.<sup>12</sup> A potential mechanism of resistance could be the loss of HGF/cMET regulation by VEGF-A, which would lead to uncontrolled HGF-regulated growth in spite of VEGF-A inhibition.<sup>13</sup> In this scenario, utilizing a cMET inhibitor would be effective in overcoming resistance to bevacizumab.

Overall, we discovered cross-talk between upregulated and activated angiogenic pathways in VS, namely the VEGF-A

and HGF pathways. Specifically, we found that siRNA-mediated knockdown of VEGFA led to a decrease in cMET expression, and knockdown of MET led to a decrease in VEGF-A and VEGFR2 levels in SCs and VS cells. Our findings are in agreement with previous work that outlines these interactions in other cell types, such as endothelial cells. Through establishing cross-talk between VEGF-A and cMET, 2 molecules typically studied independently in VS, our work can provide new ways to understand and manipulate VS pathobiology. With this novel understanding, we can design more effective pharmacotherapies, including combination therapies targets VEGF-A and cMET.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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